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TAXONOMIC STUDY ON THE GENUS *PICHIA* PHAFF (1)

KENKICHI KODAMA, TADASHI KYONO er SKOJIRO KODAMA

Laboratory of Kodama Brewing Co. Ltd.

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INTRODUCTION

One hundred and four cultures belonging to Genus *Pichia* PHAFF (*1*) have been isolated from 436 of samples (soil, fruit rind and juice of several kinds) sent from abroad (18 countries). Taxonomic study is being carried out on these fresh isolates. The purpose of our study is to obtain new or unique species together with those which, though already reported, yet seem to be taxonomically controversial, from as many samples as possible widely distributed in nature (including those which have hitherto never or scarcely been picked up), in order to serve for throwing some light on the phylogenitic problem on *Pichia* which is still unsolved in connection with diagnosis of both *Petasospora* BOIDEN et ABADIE (*2*) and *Debaryomyces* (in sensu LODDER et KREGER-VAN RIJ) (*3*) as pointed out by J. ZSOLT (*4*) J. P. VAN DER WALT (*5*) and O. VERONA (*6*).

METHODS AND EXPERIMENTAL RESULTS

The isolation of yeasts has been carried out mainly by the procedure of WICKERHAM (*7*), (*8*). For the isolation, however, the following media were employed besides those of both GRAHAM (*9*) and WICKERHAM (*7*).

Table. Special media for the isolation of Yeasts.

Medium 1 "Koji" extract

"Koji" extract can be prepared in the following way; 1 kg of "Koji" (solid culture of *Aspergillus Oryzae* on cooked ordinary rice) is packed into a cotton bag, suspended into water bath containing 4–5L of tap water, kept warm under occasional stirring at 55–56°C for 4–5 hours until starch can not be detected by iodine reaction. After taking off the bag, the extract is heated to boil, then filtrated through paper and diluted to a density of 12° Ballg. The pH is adjusted to about 4–4.5 by lactic acid.

Medium 2

Ethyl alcohol	3 % (Vol)
Ammonium sulphate	0.5 %
Potassium monophosphate	0.5 %
Magnesium sulphate	0.05%
Vitamine mixture	(LODDER's)

Medium 3

P-Oxybenzoate (Na-salt)	0.2 %
Ammonium sulphate	0.1 %
Potassium monophosphate	0.1 %
Magnesium sulphate	0.05%
Vitamine mixture	(LODDER's)

Medium 4

Glacial acetic acid	0.4 %
Ammonium sulphate	1.0 %
Potassium monophosphate	1.0 %
Magnesium sulphate	0.05%
"Koji" extract (12° Ballg)	5.0 % (Vol)

Taxonomic procedures were employed mainly by those of LODDER and KREGER-VAN RIJ (*3*) and partly by WICKERHAM (*7*) with the exception of the maximum temperature for the growth which had been regularly applied by many of classic taxonomists and is considered taxonomically significant from the view point of ecology of yeasts. Most of the isolates were identified to routine species, *Pichia membranaefaciens* HANSEN and *Pichia fermentans* LODDER group, so far as the samples firstly picked up. However, some of them seem to be noteworthy from the standpoint mentioned above. This paper deals with 3 cultures isolated from soil, fruit juice and dreg of pressed grapes, which seem closely resemble *Pichia terricola* VAN DER WALT (*10*).

DESCRIPTION

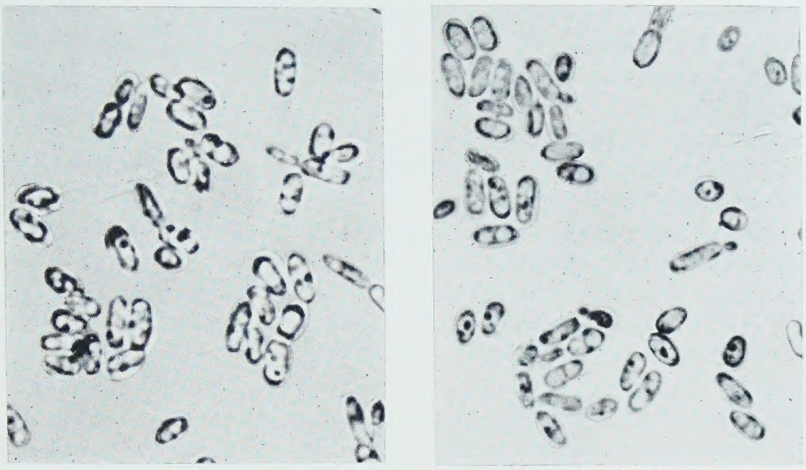
Pichia terricola VAN DER WALT form. α.

Growth in malt extract: After 3 days at 25°C cells are oval to cylindrical (2.0–6.0)×(4.0–12) μ, not so large in size as those of authentic culture, single in pairs or in short chains (Fig. 1), and a very thin, smooth, creeping pellicle is formed. After 1 month a sediment and pellicle are presented.

Streak culture on malt extract agar: After 1 mon that 17°C, the slant is yellowish white, flat, somewhat shiny almost smooth, slightly wavy at the margin.

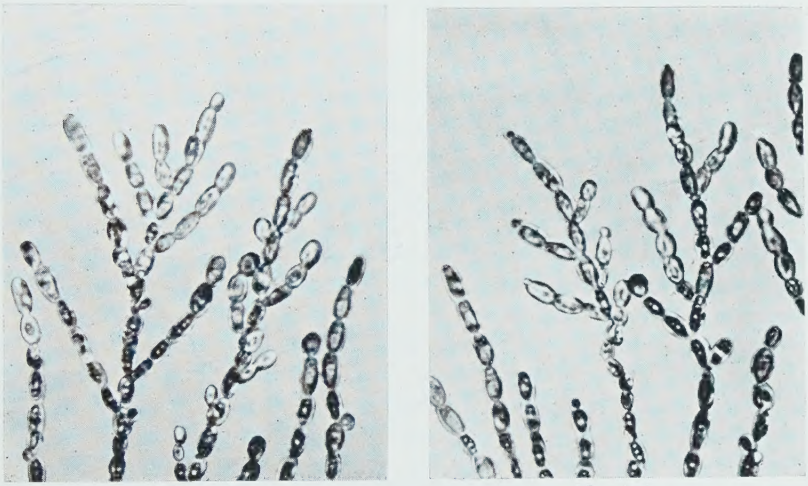
Slide cultures: Pseudomycelium in tree-like formation. There is no difference between pseudomycelium and blastospores (Fig. 2).

Sporulation: The spores are slightly oblate spherical, occasionally seem to be hemispherical with downward curving base, without any indication of brim, usually 2–4 rarely 1 per ascus with an oil drop inside (Fig. 3). No conjugation is observed immediately before ascus formation. Best sporulation is on concentrated carrot juice agar, potato juice agar with 2% glucose and malt extract-yeast extract agar slant, which is marked by the occurrence of chocolate brownish sectors due to the sporulation.



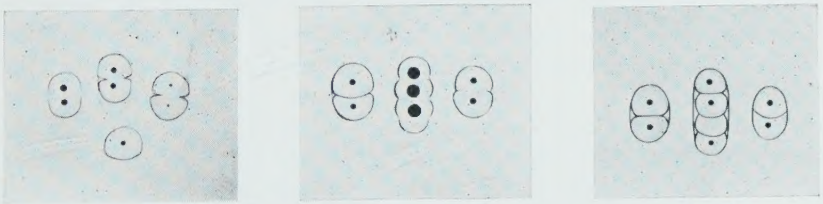
No. 009 No. 010

Fig. 1. Cells in malt extract. (×800)



No. 009 No. 010

Fig. 2. Slide cultures on potato agar. (×400)



No. 002 No. 009 No. 010

Fig. 3. Drawing of ascospores.

Fermentation: Glucose +, Galactose −, Saccharose −, Maltose −, Lactose −, Raffinose −.

Sugar assimilation: Glucose +, Galactose −, Saccharose −, Maltose −, Lactose −.

Assimilation of potassium nitrate: absent.

Ethanol as the sole source of carbon: growth, a thin pellicle is formed.

Splitting of arbutin: negative.

Vitamin requirement: Added vitamins are required.

Maximum temperature for the growth: 39–40°C.

Sources of isolation: 3 strains 002, 009 and 010-LKB were isolated from West Indian cherry juice (Sau Paulo in Brazil), soil (Pisa in Italy) and dreg of pressed grapes (Lucca in Italy) respectively.

DISCUSSION

As above mentioned, 3 cultures of film forming yeast do not assimilate potassium nitrate, these strains can be classified to Genus *Pichia*. Further, the cultures under disucssion resemble *Pichia terricola* VAN DER WALT (*10*) in the following properties.

i) Spores are roundish without any indication of brim.

ii) Fermentation and assimilation of glucose only.

iii) Streak culture on malt agar is rather shiny and marked by occurrence of brown sectors due to sporulation just as observed in nearly almost all cultures belonging to *Pichia membranaefaciens* group. According to J. P. VAN DER WALT, vegetative cells of *Pichia terricola* are considerably larger than those of *Pichia fermentans*, and based on both of the size of cells and feature of streak culture above mentioned "*Pichia terricola*" was proposed for the new species.

However, compared with descriptions of *Pichia terricola* and *Pichia fermentans* by VAN DER WALT (*10*) and LODDER (*3*), respectively, there seems to be a remarkable difference in the shape of ascospores between both species, that is, spores of the former are spherical without any indication of brim, which fact was also observed electron microscopically by KREGER-VAN RIJ on the culture of 002-LKB, deposited in the Yeast Division of the Central bureau voor Schimmel cultures (*11*), whereas, those of the latter are usually hat-shaped or semispherical with indication of short brim. Further more, the same difference could be clearly observed also between the spores of our fresh isolates, both spores bearing the respective identification. Therefore, in the author's opinion, it should be assured that remarkable characterisitics of both species are manifest in the different shapes of ascospore, along with morphological properties pointed out by the original author. Further comparative study with our isolates and the authentic culture of *Pichia terricola* revealed that some differences lie in morphological properties to follow:

i) In the author's isolates the size of cells are not so larger as that of *P. terricola*, but rather as small as the size of *P. fermentans*.

ii) In our isolates, though the shapes of ascospore may be expressed as "spherical" or "roundish", yet occasionally it seems to be somewhat oblate, or even semishperical with downward curving base as shown on Fig. 3. On the other hand, according to the original description of *P. terricola*, the shapes of ascospore were reported merely as "spherical", but unfortunately not illustrated in detail with any sketch or photograph, while the authentic culture sent from J. P. VAN DER WALT has already turned out asporogenous, so the authors conld not make any definite conclusion on whether or not the spores of *P. terricola* are different from those of our isolates in the shapes.

Eventually, taking the proposal of J. P. VAN DER WALT into consideration together with the experimental results by the authors for three of fresh isolates, the authors wish to propose a provisional denomination *Pichia terricola* VAN DER WALT form α as a new type of the authentic species.

SUMMARY

In order to obtain new or unique species belonging to Genus *Pichia* Phaff including those already, reported, which seem to play an important role in solving phylogenetic problem on yeasts, many cltures have been isolated from various kinds of samples sent from abroad. This paper is the first report which deals with three cultures selected from many of fresh isolates mentioned above, for which the authors wish to propose provisionally the name *Pichia terricola* VAN DER WALT form. α as distinguished from the authentic description in some morphological properties.

ACKOWLEDGEMENT

The authors wish to express their hearty thanks to Mrs. KREGER-VAN RIJ who gave kind assistance to their study, as well as for Professor O. VERONA, H. J. PHAFF, Rodolpho de Camargo, A. Chaves Batista and many of yeast researchers abroad who have kindly made available many samples of various kinds for the isolation of yeasts.

Further, the authors are greatly indebted to Dr. J. P. VAN DER WALT for his kindness in sending us the authentic culture of *Pichia terricola*.

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ACTIONS OF SURFACE ACTIVE AGENTS UPON LACTIC ACID BACTERIA. THE GERMICIDAL ACTION AND THE EFFECT ON THE PERMEABILITY

AKIRA ÔBAYASHI

Faculty of Agriculture, Kagoshima University

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INTRODUCTION

A large number of works have so far been done on the mechanisms of the germicidal action of surface active agents, in which several of the mechanisms have been proposed (*1*, *2*), such as denaturation of the bacterial proteins and enzymes, interference with osmotic balance in such a manner as to release internal substances outward through the cell wall, and disruption of the permeability of the bacterial cells. As yet no satisfactory explanation has been developed.

It was found in the previous investigation (*3*) that the penetration of lactate into the intact cells of lactic acid bacteria was stimulated and free amino acids were released from internal environment of the cells by the addition of cetyltrimethyl ammonium bromide.

In this paper, the germicidal action of many surface active agents on lactic acid bacteria was studied in relation to the effect on the permeability of the cells. And the mechanism of the germicidal action was discussed.

MATERIALS AND METHOD

Strain: *Lactobacillus plantarum* 11, *Lactobacillus saké* 012, *Leuconostoc mesenteroides* B07 and *Pediococcus hennebergi* 253 were used.

Surface active agents: They are shown in Table 1.

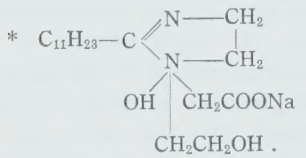
Intact cells: The organisms were grown in the medium containing polypeptone 0.5%, yeast extract 0.3% and glucose 3.0% or calcium gluconate 2.0% for 48 hours at 30°, and the cells were harvested by centrifugation, then washed three times with distilled water.

Cell-free preparation of enzymes: Wet cells were ground with quarz sand, suspended in M/30, pH 6.8 phosphate buffer and centrifuged at 15,000 g for 10 minutes.

Germicidal assay of surface active agents: To the suspension of intact cells (about 10^5 cells/ml of pH 6.8 M/30 phosphate buffer) each surface active agent was added to the various concentrations. After 5 minutes at 25°, the mixture was diluted, then the viable cells were counted in plate

Table 1. Surface active agents.

Signs of surfactants	Kinds	Structures
N-1	nonionic	polyoxyethylene lauryl alcohol ether
N-2	〃	polyoxyethylene alkyl phenol ether
CTAB	cationic	cetyltrimethyl ammonium bromide
CPC	〃	cetylpyridinium chloride
A-1	anionic	sodium laurylalcohol sulfate
A-2	〃	sodium lauryl benzen sulfonate
Am-1	amphotelic	sodium N-stearyl alanine
Am-2	〃	alkyl imidazole carbonate*



method.

Assay of dehydrogenases: Activities of dehydrogenases were assayed using 2, 6-dichlorophenol indophenol as a hydrogen acceptor. To enzyme preparation or cell suspension, 0.1 ml of 5×10^{-2} M of substrate (D-, L-lactate (Na-salt), glucose, glucose-6-phosphate (Na-salt), and gluconate (Ca-salt)), 0.2 ml of pH 6.8 M/30 phosphate buffer and 0.1 ml of 5×10^{-4} M of 2, 6-dichlorophenol indophenol were added, then the reaction mixture brought to 0.8 ml with water. Activity of enzyme was indicated by the length of time it takes to decolorize the pigment at 35°.

Determination of lactic acid: The method proposed by Barker and Summerson (*4*) was used.

Determination of amino acids: Ninhydrin method using alanine as standard was used.

EXPERIMENTAL RESULTS AND DISCUSSION

A) Germicidal power of surface active agents

The germicidal power of various surfactants for lactic acid bacteria was shown in Table 2. Cationics were most powerful germicide and nonionics did not show any action on all strains used.

B) The relation between germicidal power and its effect on the permeability to lactates and on the release of internal substances from the cells.

As reported in the previous paper (*3*), the penetration of the substrate follows the rate limiting for lactic dehydrogenase activity of the intact cells of *L. delbrückii*. And by the addition of CTAB to the intact cells the

Table 2. The germicidal power of surfactants to various lactic acid bacteria.

Strains \ Surfactants	N-1	N-2	CTAB	CPC	A-1	A-2	Am-1	Am-2
L. plantarum 11	—	—	##	##	+	+	+	+
L. sake 012	—	—	##	##	##	##	+	##
Leuc. mesenteroides	—	—	##	##	+	+	+	+
Pc. hennebergi	—	—	##	##	+	+	+	+

##: Germicidal action was observed at 0.1 mg/ml of the concentration of surfactant.
+: at 1.0 mg/ml.
—: not observed at 1.0 mg/ml.

Table 3. The effect of surfactants on the intact cells of *L. plantarum*.

Surfactants added	Activity of D-lactic dehydrogenase (5×10^8 cells*)			Germicidal power (viable cells)		Substances released	
						Amino acids (as alanine)	Adsorption at 260 mμ***
	50 μg		200 μg	50 μg/ml	1,000 μg/ml	5 mg	
	m. s.		m. s.				
—**	7. 30			10^5		150 μg	0.060
N-1	7. 00		7. 20	10^5	10^5	240	0.095
N-2	6. 20		6. 00	10^5	5×10^3	400	0.158
CTAB	2. 00		2. 40	0	0	1,260	0.442
A-1	5. 30		8. 00	10^4	0	1,060	0.583
Am-2	3. 40		4. 00	10^4	0	700	0.240

Determination of the substances released: To the intact cells (about 40 mg dry weight) 5 mg of surfactant was added, then total volume was brought to 2 ml with water. After 3 hours at 25°, the cells were took off by centrifugation. And free amino acids and the substances which have ultra-violet adsorption in the supernatant were measured.

* The cells which used for the assay of enzyme.
** Without surfactant.
*** Supernatant was diluted to 60 times, then measured the optical density at 260 mμ. (light path 1 cm).

enzyme was activated as the result of stimulation occasioned by the penetration of lactate. From this it should be possible to investigate the effect of surfactant on the permeability of intact cells to lactate by the change in the activity of lactic dehydrogenase.

Moreover, it was found that free amino acids and the substances which have ultra-violet adsorption were released from internal environment of the cell by the addition of CTAB.

Table 4. The effect of surfactants on the intact cells of *L. saké*.

Surfactants added	Activity of L-lactic dehydrogenase (2×10^8 cells)		Germicidal power (viable cells)	Amino acids released (as alanine)
	25 μg	100 μg	200 μg	5 mg
	m. s.	m. s.		
—	5. 00		10^5	120 μg
N-1	5. 00	5. 10	10^5	160
N-2	3. 40	3. 00	10^4	220
CTAB	0. 10		0	790
A-1	2. 50	0. 30	2×10^2	740
Am-2	2. 50	0. 20	0	510

Table 5. The effect of surfactants on the cell-free lactic dehydrogenase of *L. plantarum*.

	Activity of dehydrogenases	
	D-lactic	L-lactic
	m. s.	m. s.
Cell-free enzyme	1. 40	3. 30
〃 +N-1 200 μg	1. 40	3. 30
〃 +N-2 200	1. 40	3. 30
〃 +CTAB 50	1. 40	3. 30
〃 +A-1 100	4. 20	3. 50
〃 +Am-2 200	1. 50	3. 30

These actions of surfactants were measured in relation to the germicidal power with the intact cells of *L. plantarum* and *L. saké*.

These results were shown in Tables 3 and 4. The enzyme stimulating effect of surfactant seems to be proportional to its germicidal power. And also remarkable parallelisms between the release of internal substances and the germicidal power were observed to be in agreement with the result reported by HOTCHKISS (*2*). Anionics inhibits cell-free lactic dehydrogenase, but the other surfactants hardly produce any effect (Table 5). Therefore, as reported in the previous paper (*3*) the increase in enzyme activity of intact cells by the addition of surfactants could be regarded as the increase in the penetration of lactate.

C) The effect on the penetration of metabolized substances into the cells.

The effect on the penetration of lactates which are final products of lactic acid bacteria has already been investigated. Here, it was studied on the penetration of the metabolized substances, such as glucose, gluconate

and glucose-6-phosphate.

(i) *Lactobacillus plantarum* 11

Incubating the intact cells of *L. plantarum* with glucose, gluconate or glucose-6-phosphate and surfactants, the effect of surfactants on the formation of lactic acid was studied. The results presented in Table 6 illustrate that glycolysis of intact cells was inhibited by the germicidal surfactants, but glycolysis of cell-free preparation was not inhibited by surfactants except anionics. From these results, it may be suggested that the penetration of glucose into the intact cells is inhibited by germicidal surfactants.

Table 6. The effect of surfactant on glycolysis by *L. plantarum*.

Substrates	Surfactants added	Lactic acid formed		
		Cells grown in glucose medium		Cells grown in gluconate medium
		Intact cells	Cell-free preparation	Intact cells
Glucose	—	205 μg	29 μg	185 μg
〃	—*	—	58	—
〃	N-1 150 μg	229	29	205
〃	CTAB 25	0	32	15
〃	〃 50	0	30	0
〃	A-1 150	156	12	76
〃	Am-2 150	86	27	15
G-6-P**	—	0	67	0
〃	CTAB 25	0	68	0
Gluconate (Ca-salt)	—	0	0	82
〃	CTAB 25	0	0	0

Reaction mixture: Cell suspension 0.1 ml (corresponded to 5×10^9 cells), or cell-free preparation 0.1 ml (corresponded to 7×10^{10} cells), pH 6.8 M/20 phosphate buffer 0.4 ml, substrate 2 μM and surfactant.

Total volume was brought to 1.0 ml with distilled water.

Reaction period: 1 Hour.

Reaction temperature; 30°.

* 0.5 μM of adenosine triphosphate was added to the reaction mixture.

** Glucose-6-phosphate (Na-salt).

Similar inhibitory effect of surfactants was observed in the intact cells grown in the medium containing 2% gluconate (Table 6). Different from the intact cells obtained in the glucose medium, however, gluconate grown cells, since they incorporate glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (5), are able to metabolize gluconate. Therefore, in the similar method followed in lactic dehydrogenase the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

of the intact cells grown in the gluconate medium were measured by adding surfactants. For the measurement of glucose-6-phosphate dehydrogenase, glucose was used for substrate, on the ground that glucose-6-phosphate seems unable to penetrate into the cells, as shown in Table 6. The inhibitory effect of germicidal surfactant was observed as presented in Table 7, indicating that the germicidal surfactants seems to inhibit the penetration of these metabolized substances.

(ii) *Leuconostoc mesenteroides* B07

As shown in Table 8, the cells of *Leuc. mesenteroides* which have glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

Table 7. The effect of surfactants on the various dehydrogenases of *L. plantarum*.

	Activities of dehydrogenases						
	Intact cells (5×10^8 cells)			Cell-free preparation (corresponded to 5×10^9 cells)			
Substrates / Surfactants added	D-Lactate	Glucose	Gluconate	D-Lactate	Glucose	G-6-P	Gluconate
	m. s.	m. s.	m. s.	m. s.	m.	m.	m.
—	7. 30	1. 30	1. 30	1. 40	50 (20)	15	40 (3)
N-1 200 μg	7. 00	2. 00	2. 00	1. 40	—	15	(3)
CTAB 25	2. 30	∞	∞	—	—	—	—
〃 50	2. 00	∞	∞	1. 40	—	16	(3)
A-1 200	5. 30	3. 00	4. 20	∞	—	∞	(13)
Am-2 200	3. 40	4. 00	5. 00	1. 50	—	16	(3)

The figure put in bracket shows the decolorization time on the addition of 0.5 μM of adenosine tripeosphate.

∞ means no decolorization after 60 minutes.

Table 8. The effect of surfactants on the various dehydrogenases of *Leuc. mesenteroides*.

	Activities of dehydrogenases	
Substrates	D-Lactate	Glucose
	m. s.	m. s.
Intact cells (2×10^8 cells)	6. 00	1. 10
〃 +N-1 200 μg	6. 00	1. 20
〃 +CTAB 50	5. 00	∞
〃 +A-1 100	7. 00	7. 00
〃 +Am-2 200	6. 00	∞
Cell-free preparation (corresponded to 2×10^9 cells)	2. 40	7. 00*
〃 +CTAB 50	2. 40	7. 00*

* With 0.5 μM of ATP.

constitutively, revealed almost same result with the cells of *L. plantarum*.

D) Difference of the effect of CTAB on the permeability between strains

Lactic dehydrogenase of the intact cells of *L. plantarum* as well as *Leuc. mesenteroides* was activated by the treatment with toluol and lyophilyzation. On the other hand the enzyme of the cells of *Leuc. mesenteroides* was not activated by the addition of CTAB, different from the cells of *L. plantarum* (Table 9). It is probable that each strain specifically reacts in the premeability to lactate upon addition of a certain surfactant.

Table 9. Increase in the activity of D-lactic dehydrogenase by various treatments of intact cells.

Strains	Activity of D-lactic dehydrogenase	
	L. plantarum	*Leuc. mesenteroides*
	m. s.	m. s.
Intact cells	7. 30	6. 00
〃 +CTAB 50 μg	2. 00	5. 00
Toluol treated cells	2. 20	2. 30
〃 +CTAB 50 μg	2. 00	6. 00
Lyophilized cells	2. 30	2. 10
〃 +CTAB 50 μg	2. 10	5. 30

Each cells used for assay were about 5×10^8 cells in *L. plantarum*, and 2×10^8 cells in *Leuc. mesenteroides*.

E) Analysis on the mechanism of the germicidal action of CTAB

By adding cationics CTAB and anionics A-1 simultaneously to the intact cells of *L. plantarum*, the activity of lactic dehydrogenase, the formation of lactic acid and viable cells were measured. The data summarized in Table 10 show that the action of CTAB is prevented by almost equal mole of A-1, suggesting the combination of CTAB with A-1.

Table 10. The effect of the simultaneous addition of CTAB and A-1 on the intact cells of *L. plantarum*.

Surfactants added							
	CTAB μg	—	25	25	25	50	—
	A-1 μg	—	—	25	50	25	50
Activity of D-Lactic dehydrogenase	m. s.	12.00	3.30	12.00	12.00	4.20	12.00
Glycolysis (lactic acid formed)	μg	275	0	205	214	0	242
Viable cells	cells	5×10^8	4×10^4	4×10^8	4×10^8	4×10^4	5×10^8

Glycolysis: Details of the assay for glycolysis were given in Table 6.

Intact cell suspension

—added with CTAB corresponded to 25 μg/5$\times$10^8 cells

Cell suspension A

—centrifuged immediately

Cells

—suspended in pH 6.8 M/20 phosphate buffer

Cell suspension B

—added with A-1 corresponded to 30 μg/5$\times$10^8 cells

—shaking vigorously for 30 seconds

—centrifuged immediately

Cells

—suspended in pH 6.8 M/20 phosphate buffer

Cell suspension C

Figure 1. Washing of CTAB-treated cells of *L. plantarum* with A-1.

Table 11. The effect of washing of CTAB-treated cells with A-1.

	Activity of D-lactic dehydrogenase	Glycolysis (lactsc acid formed)	Viable cells
	m. s.	μg	
Intact cell suspension	6. 30	275	5$\times$10^8
〃 +CTAB 25 μg	2. 30	0	
Cell suspension A	3. 10	0	
〃 +CTAB 25 μg	2. 50	-	
Cell suspension B	2. 40	0	6$\times$10^4
〃 +CTAB 25 μg	2. 40	—	-
Cell suspension C	5. 00	53	7$\times$10^7
〃 +CTAB 25 μg	3. 00	0	
Cell suspension C*	7. 30	0	0
〃 +CTAB 25 μg	4. 30	—	

* Cell suspension washed with A-1 after 20 minutes of treatment with CTAB.

Following the procedure shown in Figure 1, immediately after treatment with CTAB, cells were washed with A-1, and the activity of lactic dehydrogenase and glycolysis and the count of viable cells were measured with the same concentration of the cell-suspension at each step (Table 11). These effects of CTAB on the activity of lactic dehydrogenase, lactic acid

formation and growth (viz. colony formation) were clearly reversed by washing the cells with A-1. However, when the washing with A-1 was delayed, say 20 minutes, such reversal was not observed.

In the previous paper (*3*), it was proposed that CTAB bind tightly with the cells. Therefore, it is supposed that the cells which bind with CTAB is not killed but unable to grow as a result of change in the permeability, and by the continuous binding with CTAB the cells release the internal substances step by step and finally die off.

ACKNOWLEDGEMENT

The author wishes to acknowledge the valuable advice and kind criticism of Prof. K. KITAHARA, University of Tokyo and Prof. M. KANIE, Kagoshima University.

SUMMARY

The mechanism of the germicidal action of surface active agents was studied. It was observed that surfactants have the following action on intact cells of lactic acid bacteria; (1) accelerate the penetration of lactate into the intact cells, (2) liberate the free amino acids from internal environment, (3) prevent the penetration of glucose and gluconate, which are metabolic sources of energy for the cell.

Some remarkable parallelisms between these actions and their germicidal power were observed.

When the cells which had changed in the permeability and been unable to grow by binding with CTAB were washed with the oppositely charged surfactant A-1. The permeability of the cells reversed to that of the intact cells and the cells grew again.

From these results, it is supposed that the germicidal action of surfactants depends on the action to immediately bind with the cells changing the permeability to unsuitable one for growth.

Moreover, anionic surface active agents inhibit lactic dehydrogenase and glucose-6-phosphate dehydrogenase. It seems likely that the germicidal effect of anionics depends not only on the action to change the permeability of the cells but also on the action to denaturate the cell proteins.

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DNA-MEDIATED TRANSFORMATION IN *BACILLUS SUBTILIS* WITH SPECIAL REFERENCE TO THE METHOD FOR PREPARING COMPETENT CELLS

HIUGA SAITO, MASAMICHI KOHIYAMA AND
YONOSUKE IKEDA

Institute of Applied Microbiology, University of Tokyo, Tokyo

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Since SPIZIZEN reported DNA-mediated transformation in *Bacillus subtilis* in 1958[1], many investigators have focused their attention on this phenomenon and evaluated the method as a promissing way for studying fundamental genetics. If one wishes to study transformation through this system, however, one will encounter many difficulties with respect to the technique for preparing bacterial cells which are competent for transformation. In the present paper, some fundamental informations necessary for conducting transformation experiments are given.

MATERIALS AND METHODS

Strains: The mutant stocks of *B. subtilis* Marburg strain used in these experiments were originally supplied by the courtesy of Dr. D. M. BONNER, Department of Microbiology, Yale University. Strain No. 116 (nicotinic acid dependent) was used for DNA preparation. Strains No. 160 S^r (tryptophan dependent and streptomycin resistant) and No. 168 (indole or tryptophan dependent) were employed as recipients. These strains were stocked on sporulation medium in the cold.

Media: Sporulation medium was prepared from potatoes. Five hundred grams of washed potatoes were chipped and extracted with 1 liter tap water at 120° for 10 min. The extract was filtered through cheese cloth (Gauze) and pH was adjusted to 7.0 after 5 mg per l. of $MgSO_4 \cdot 7H_2O$ was added. Fifteen gram agar was added to 1 l. of the filtrate to make slanted media.

Nutrient broth consisted of 10 g meat extract, 10 g polypeptone, 1 g yeast extract (Difco), 1 g NaCl and 1 l. tap water; pH was adjusted to 7.0 before autoclaving.

Minimal medium (MM) comprised 7 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g Na_3-citrate$\cdot 2H_2O$, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 g glucose and 1 l. distilled water. Solutions of magnesium sulfate, other salts, and glusose were autoclaved separately and mixed together after cooling. Solid medium is prepared by adding 15 g (per liter) Difco agar dissolved in water to concentrated liquid

medium. Dipottasium phosphate made in Japan was occasionally impure, and it was found better to substitute it with 5.46 g KH_2PO_4 and 2.26 g KOH. These cautions are necessary to avoid precipitation which lowers the efficiency of transformation. Twenty ml. of the solid medium was plated in a Petri dish and was placed overnight at 50° before use..

Supplemented media: The incubation of recipient cells were carried out in MM containing either yeast extract or casamino acids (Difco), *i.e.* MYI:yeast extract 0.1%, MYII:yeast extract 0.01%, MCI:casamino acids 0.1% and DL-tryptophan 50 γ/ml., MCII:casamino acids 0.01% and DL-tryptophan 0.5 γ/ml. Auxotrophs were cultivated in MM supplemented with 30 γ/ml DL-tryptophan, 10 γ/ml indole, or 0.5 γ/ml nicotinic acid.

These media were sterilized under a modest condition, *i.e.* heated for 15 min. at temperature ranging from 100° to 120° in a gas-autoclave and cooled as quickly as possible.

Preparation of DNA: Cells of 116 *nic*, which had been incubated in nutrient broth for 12 hours at 30° on a reciprocal shaker, were washed twice with 0.05 M phosphate buffer (pH 7.2) and suspended in the same buffer containing 20% sucrose. The cell suspension was then treated with 0.5 mg/ml lysozyme for 1 hour at 37°. The resultant protoplasts were collected by centrifugation at 15,000×G for 20 min. and transferred to saline containing 0.015 M Na_3-citrate to give osmotic shock. This was followed by centrifugation at 17,000×G for 20 min. The precipitates were extracted with 10% NaCl in the presence of Na_3-citrate (1%). The crude extract was then deproteinized by shaking with chloroform and octanol, followed by the separation of water layer by centrifugation; this process was repeated at least four times until the presence of denatured proteinous layer became negligible. The DNA solution was treated with 20 γ/ml RNase for 5 min. at room temperature and the resultant was deproteinized again. From the purified extract highly polymerized DNA was precipitated by the addition of two volumes of EtOH. The precipitate was stored in 60% EtOH in the cold, and dissolved in 10% NaCl containing 1% Na_3-citrate when used for transformation experiments. The concentration of DNA was determined spectrophotometrically; O.D. 0.022 at 260 mμ corresponds to 1 γ DNA/ml.

Incubation of recipient cells: Approximately 5×10^9 spores were suspended in one ml of the supplemented media and heat-shocked at 60° for 5 min. Recipient cells were incubated in two steps: 1st and 2nd cultures. Incubation was carried out in a L- or a reverse T-type tube on a see-saw type (Monod's) shaker at 37°. DNA was added to the 2nd culture. The final concentration was approximately 30 γ/ml unless otherwise stated. After an appropriate period, the cell-DNA mixture was added in the cold to 1/50 volume of DNase solution (crystalline DNase 500 γ/ml) containing 10^{-2} M $MgSO_4$. One tenth ml aliquots were sperad on MM immediately after the addition of DNase. Plating was made in triplicate and number of colonies appeared on the plates after two days incubation at 37° were scored as transformants. Under the experimental conditions, the number

of transformants which appears during a certain period is indicative of a function of the number of competent cells. Deviation in triplicate plates is less than $\pm 10\%$.

Growth of cells was determined by the KOTAKI's nephthalomometer using visible light and expressed in terms of the units of Coleman's nephelos standard. The nephelos value (NV) reflects the number of cells under experimental conditions better than O.D.; the linear correlation between NV and O.D. holds in the range from 0 to 600 NV (Figure 1). A culture of 1000 NV cotains approximately 4×10^8 cells/ml.

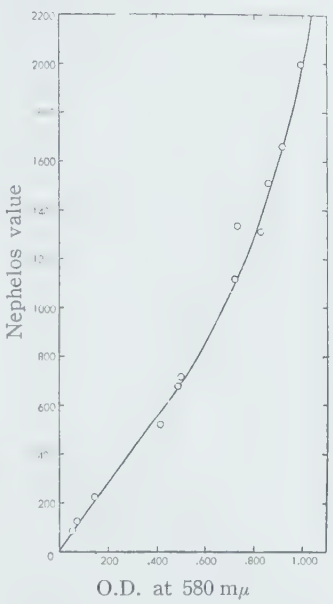

Fig. 1. Correlation between nephelos value and optical density of bacterial culture. Data were collected from the 1st cultures in MYI medium.

RESULTS AND DISCUSSIONS

1) *Optimal incubation period on the 1st culture.*

The method described in the report of SPIZIZEN[1] was slightly modified. Approximately 2×10^7 spores/ml of 160 S^r or 168 were cultivated in the MYI medium (1st culture). At intervals, cells were harvested and transferred to two volumes of MYII medium. After addition of transforming DNA the cell-DNA mixture was incubated for another one hour on the MONOD type shaker. Transformants were detected by plating aliquots onto MM after DNase treatment.

Growth of cells during the 1st culture was a typically exponential one following 1–2 hour lag period (Figure 2). Microscopic observation exhibited peculiar pleiomorphism. In the early period, germinated spores elongated without cell division and formed long mycelium-like cells which when stained, appeared to comprise a large number of normal rod cells. These long cells then divided into rods before the growth curve reached stationary phase. It was found that the transformation occured at the highest frequency when the cells terminating exponential growth were transferred to the 2nd culture (Table 1). The best incubation period of the 1st culture was around 5 hours.

Between the 160 S^r and the 168 which were used as recipients, the former always gave higher frequencies of transformation. Therefore 160 S^r was used throughout the following experiments.

When MCI medium was used instead of MYI, the germination of spores and subsequent growth were extremely retarded. The optimal inducubation period for transformation of this culture was 6–7 hours (Figure 2, and

Table 1). As was described in recent paper[2] vegetative cells harvested from a overnight culture in Penassay or nutrient broth could be used as inoculum for the 1st culture. These cultures exhibited the highest competence at 4–5 hours but the maximum frequencies of transformation were far lower than those of the spore cultures described above.

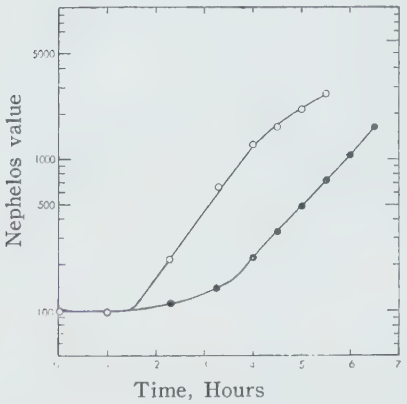

Fig. 2. Growth curves of 1st cultures inoculated with spores. Heat-shocked spores were inoculated (2.2×10^7/ml) into MYI (open circles) or MCI (closed circles), and incubated at 37°.

2) *Conditions affecting transformation in the 2nd culture.*

Cells harvested from the 5 hour-1st culture in MYI medium were resuspended in 2 volumes of MYII medium and incubated at 37°. The size of inocula was approximately 3×10^8 cells per ml. DNA was added at various times during the 2nd culture and the time courses of transformation were examined. As shown in Figure 3, the number of transformants increased linearly with the time lapse after a short induction period. The velocities of transformation at the linear portions as well as the lengths of induction periods were different according to the time of DNA addition. Cells acquired the highest competence at 30 min. incubation when the growth became slower. These results indicate that a large number of competent cells are present in the

Table 1. Transformation with cells harvested from various stage of growth in the 1st culture.

Experim. No.	Medium used in the 1st culture	Nr. of transformants in the 2nd culture, per 0.1 ml							
		Time of cell harvest (hours)							
		3	4	4.5	5	5.5	6	6.5	7
1	MYI	50	300		6600				
2	〃		142		13900		4600		
3	〃				1600		5000		1000
4	〃	0	6		145				
5	〃			250	620	40			
	MCI				130	660	1100	2460	

160 S^r were used recipients except for experiment 4 where 168 was used. Cells harvested from the 1st cultures were mixed with DNA for 60 min. in MYII (MCII for cells from MCI). Experiments 1-4 were carried out at the Dept. of Microbiology, Yale Univ. Conn.

population at the early stationary phase and that the process of transformation is independent of cell growth.

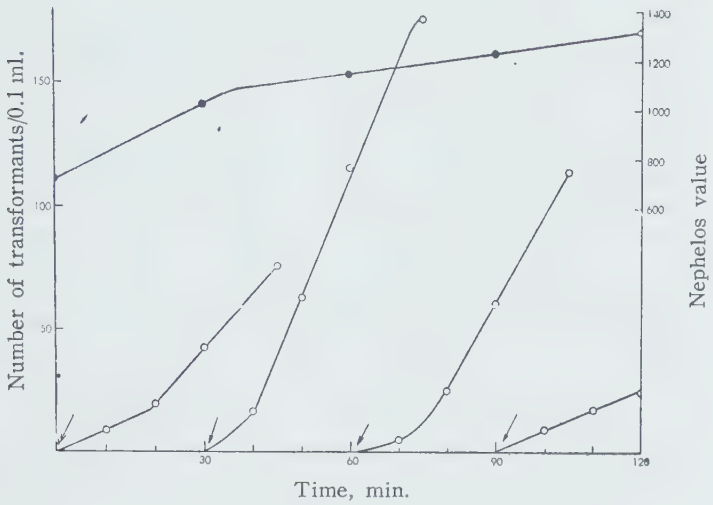

Fig. 3. Time courses of transformation in the 2nd culture. Cells harvested from the 5 hour-1st culture were incubated in 2 volumes of MYII. DNA was added at the time indicated by arrows. Aliquots removed at various times were plated on MM after treatment with DNase for the detection of transformants (open circles). Closed circles are the nephelos values during the 2nd culture.

The yeast extract in the MYII medium was found effective for the acquisition of competence but rather inhibitory for the occurrence of transformation. This is shown in the experiments in Table 2. The cells in this

Table 2. Effect of supplements in the medium on transformation

Supplement	None	Yeast extract	Casamino-acids	Tryptone	Not resuspended
Number of transformants, per 0.1 ml.	318	149	295	119	315

Competent cells were harvested from the 2nd culture at 30 min. and resuspended in MM with or without supplements (0.01% for each). After 15 min. incubation with transforming DNA, aliquots are treated with DNase and plated on MM for detecting transformants.

experiment were harvested at 30 min. in the 2nd culture and resuspended in the same volume of MM with or without supplements. DNA was added to these media. Transformation occurred at the highest frequency in MM was comparable to that of the control runs where the recipient cells without being resuspended were mixed with DNA in MYII. It seemed that the

inhibitory effect of the supplement in the 2nd culture was eliminated during the 30 min. incubation.

Yeast RNA digested with pancreatic RNase was found to produce similar or, sometimes more drastic, inhibitory effect than yeast extract. Although casamino acids had no effect under conditions described in Table 2, this substance also exhibited inhibition when tested under different conditions. Glucose is essential for the completion of transformation.

Acquisition of the competence for transformation was affected by the size of inocula in the 2nd culture. Ten to 50 times dilution with MYII medium instead of twice was found to be better in terms of the frequency of transformation per unit volume of culture (Table 3). In the diluted cultures, however, cells acquired the maximum competence after a longer cultivation, and moreover, the number of transformants did not increase linearly. Therefore studies were mostly carried out under conditions giving large population of cells in the 2nd culture.

Table 3. Transformation experiments with the diluted inocula

Dilution rate*		$\frac{1}{2}$	$\frac{1}{10}$	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{1000}$
Nephelos value (2nd cult.)	Initial	742	103	22	12	6
	Final (110 min).	1240	270	57	56	33
Nr. of transformants	per 0.1 ml.	21	914	136	63	62
	per 10^6 cells	0.4	91	68	63	310

* Cells harvested from the 5 hr-1st culture (8.0×10^8 cells/ml) were inoculated in 2-1000 volumes of MYII.

DNA was added at 60 min. after the initiation of the 2nd cultures and the cell-DNA mixtures were incubated for another 50 min.

3) *Concentration of DNA.*

It appears that the velocity of transformation depends on the concentration of DNA as well as on the competence of the recipient cells. Figure 4 is time courses of transformation where various concentrations of DNA were used. When a large amount of DNA was used, the transformation velocity was inconstant with time and the number of transformants decreased abruptly. It may be that the DNA at the high concentration affects the competence of cells. However, the initial velocities of transformation are exactly proportional to the DNA concentrations as far as the cells of constant competence are employed (Figure 5). This result is reconcilable with the hypothesis proposed in Pneumococcal transformation[3] that the overall occurrence of transformation involves a rapid reversible absorption of the nucleic acid to the bacteria, followed by a slow irreversible incorporation.

The present data raise a question as to why the saturation concentration of DNA is so high as compared with that appeared in other papers[2,3]. This may be acribed to the higher concentration of recipient cells used in the present system.

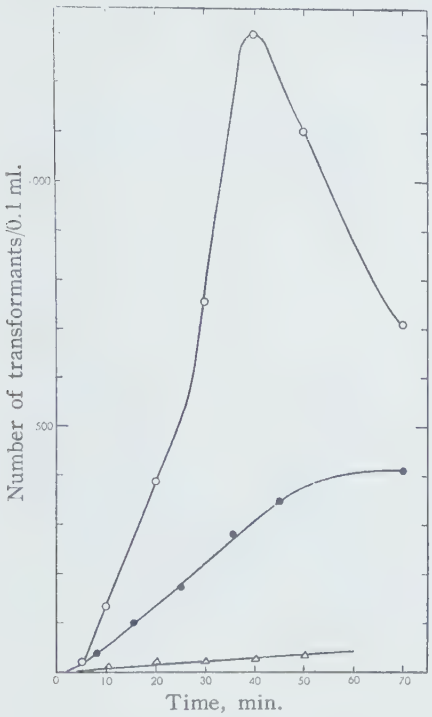

Fig. 4. Time courses of transformation with different amounts of DNA. 32γ (open circles), 16γ (closed circles), or 3.2γ (triangles) of DNA was added to one ml of 2nd culture after 30 min. incubation. Aliquots withdrawn at various times of cultivation were plated on MM after treatment with DNase.

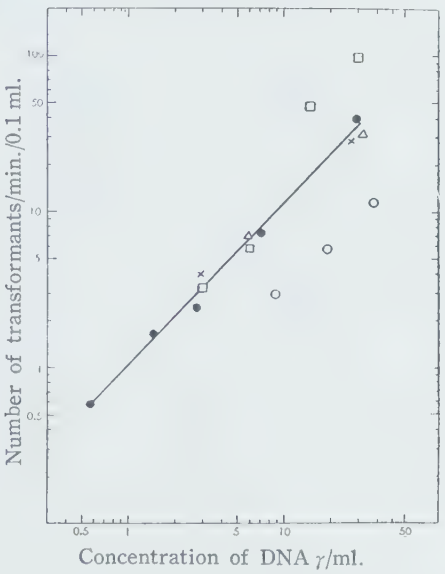

Fig. 5. Effect of DNA concentration on the rate of initiation of transformation. The different symbols represent separate experiments.

4) *1st culture in a chemostat.*

As stated in the preceding section, acquisition of competence by the recipient cells is under the control of metabolic activities at the early stationary phase of growth in the 1st and the 2nd cultures. It was thought, therefore, that better physiological conditions could be provided by controlling growth in a chemostat. A simple chemostat was designed as shown in Figure 6. The main chamber was charged with approximately 45 ml MYI medium and 1 loopful of 160 S^r cells was inoculated. Incubation was carried out with aeration. Input rate of MYI medium was controlled by a screw cock. When

the growth in the main chamber attained a steady state, cells were harvested and incubated with DNA in 2 volumes of MYII medium for 60 min. Optimal flow rate of the medium was around 5.5 ml. per hour (Table 4).

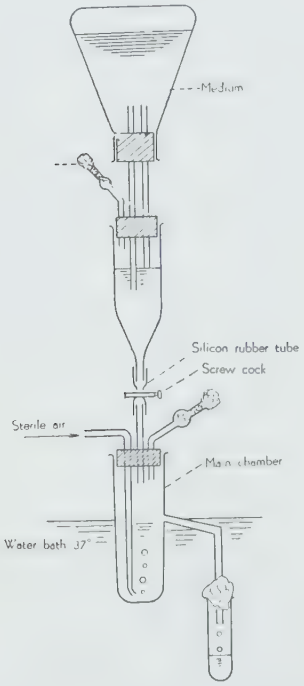

Fig. 6. A simple chemostat used for the 1st culture.

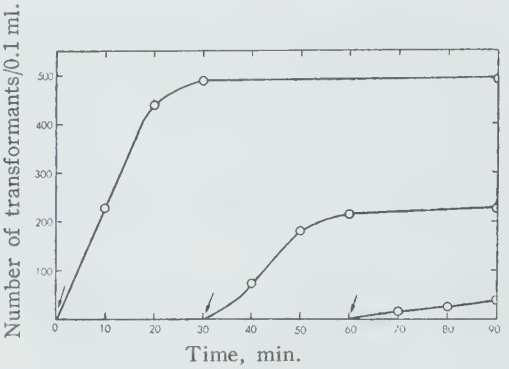

Fig. 7. Time courses of transformation in the 2nd culture inoculated with cells prepared at low temperature. Cells harvested from overnight culture in MCI at 27° were resuspended in 2 volumes of MCII and incubated at 37°. DNA was added at the times indicated by arrows.

Table 4. Distribution of competent cells in recipient cells incubated in the "chemostat"

Flow rate of medium, ml./hour	Nephelos value	Nr. of cells/ml.	Nr. of transformants, per 0.1 ml.
4.4	1440	4.6×10^8	300
5.5	1150	4.3	4020
6.3	1120	4.2	80
10.5	850	3.0	105
12.3	730	2.0	163

Cells harvested from the 1st culture were incubated in 2 volumes of MYII with DNA during 60 min.

5) *1st culture at low temperatures.*

If it is possible to prepare competent cells by overnight incubation of the 1st culture, this method would give practical convenience for schduling

laboratory works. Temperature control was found useful for this purpose. As shown in Table 5, overnight cultures in MCI at 27° had high competence for transformation.

Table 5. Distribution of competent cells in recipient cells incubated at low temperatures

Exp. No.	1st culture						dilution rate of 2nd cult.	Nr. of transformation per 0.1 ml.
	Incubn. temp.	Medium	Nr. of spores inoculated per ml.	time of incubation	final NV	Final Nr. of cells per ml.		
1	25°	MYI	2.4×10^5	14h50min.	500	2.2×10^8	1/2	0
	27°	〃	〃	〃	1260	3.4×10^8	1/4	378
	30°	〃	〃	〃	1860	5.2×10^8	1/4	4
2	27°	MYI	1.0×10^6	17:00	1520	7.6×10^8	1/4	5
	30°	〃	〃	〃	2000	8.2×10^8	1/4	0
	27°	MCI	〃	〃	400	1.0×10^7	1/2	211
	30°	〃	〃	〃	640	1.0×10^8	1/2	120
3	27°	MCI	2.0×10^7	14:15	1760	2.5×10^7	1/2	1700

Cells harvested from 1st cultures were suspended in 2 or 4 volumes MYII (MCII for cells from MCI) and incubated with DNA at 37° for 120 min. Cultures in MCI contained plenty of elongated cells; the NV and number of cells were not correlated.

Optimal conditions in the 2nd culture were somewhat different from those of the methods described in the preceding sections. Transformation occurred at the highest frequencies when DNA was added at 0 time to the 2nd culture in MCII medium (Figure 7). Addition of supplements to the 2nd culture suppressed the transformation, and MM gave a better result than MCII.

SUMMARY

A routine method of DNA-mediated transformation is described. Cells of tryptophan dependent strain (No. 160 S^r) of *Bacillus subtilis*, harvested at an early stationary phase of growth in a glucose minimal medium containing 0.1% yeast extract, are found to be the most competent for transformation (1st culture). When transforming DNA is added to the cells suspended in a fresh medium (2nd culture), transformation occurs linearly with the time lapse after a short lag period. The process of transformation seems independent of cell growth, although the acquisition of competence by the cells is under the control of delicate physiological conditions of the cells which are terminating exponential growth. The yeast extract which is required for the cell growth in the early 2nd culture, when added in excess, lowers the efficiency of transformation. The inhibitory effect of the supplemented medium is eliminated during the growth of cells. The similar inhibition is demonstrated by the addition of nucleotides or amino acids.

Physiological control of cell growth in the 1st culture by changing incubation conditions improves the competence. The use of chemostat or the incubation at low temperatures were proved to be an efficient way for obtaining competent cells.

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STUDIES ON LIGHT SCATTERING SPECTRUM OF MICROBIAL CELLS IN SUSPENSION

SHOZO KOGA and TERUYUKI FUJITA

The Institute of Applied Microbiology, University of Tokyo

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INTRODUCTION

As described in a previous paper (*1*), spherical microbial cells in suspension could approximately be regarded as optically homogeneous scatterers with total cross sections expressed by Rayleigh-Gans' or Hart-Montroll's formulae. In the present paper, it is shown that the wave-length dependence of turbidity can serve as "light scattering spectrum" for obtaining biological information on cellular level. In order to carry out the light scattering analysis of this kind with respect to microbial suspensions, a commercially available spectrophotometer could conveniently be used as shown later instead of the one mentioned in the preceding paper. General procedure of the analysis will be dealt with and the outcome will be applied to microbiological problems with special regard to *Saccharomyces cerevisiae*.

THEORY AND APPARATUS

To facilitate the discussion, the basic equations described in the preceding paper (*1*) for light scattering of spherical cells in suspension will be cited as follows with a graphical representation in Fig. 1:

$$\sigma_s/\pi a^2=(m-1)^2\phi(x)\ ,$$
$$\phi=(5/2)+2x^2-(\sin 4x/4x)-(7/16x^2)(1-\cos 4x)$$
$$+\{(1/2x^2)-2\}\{0.577+\log 4x-C_i(4x)\}\ , \qquad (1)$$
$$C_i(x)=-\int_x^\infty(\cos u/u)du\ .$$

$$\sigma_s/\pi a^2=\{\pi x^2(m-1)^2/2m\}\{(m^4+6m^2+1)\Delta_1-2(m^2+1)x^{-2}\Delta_2+x^{-4}\Delta_3\}\ ,$$
$$\Delta_j=I_j[x(m+1)]+I_j[x(m-1)]\ ,$$
$$I_1(2x)=(1/2\pi)-(1/8\pi)\{J_{1/2}^1(2x)+{}_1F_2^2(2x)\}\ ,$$
$$I_2(2x)=(1/\pi)\{\sin 4x/2x)-(\sin^2 2x/4x^2)-1+\varphi(4x)\}\ , \qquad (2)$$
$$I_3(2x)=(1/\pi)\}2x^2+x\sin 4x-(5/2)\sin^2 2x+\varphi(4x)\}\ ,$$
$$\varphi(z)=\int_0^z(1-\cos y)dy/y\ .$$

a: radius of spherical cells

n_0: index of refraction of suspending medium
n_1: index of refraction of suspended cells
λ_0: wavelength of light in the suspending medium
σ_s: cross section of light scattering

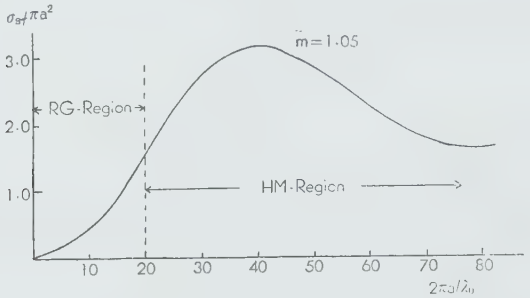


Fig. 1. Light scattering cross section for spherical scatters (relative index of refraction: 1.05).

Since index of refraction m is of values close to unity referred to the medium in all live cells, corresponding first approximate forms to equs. (1) and (2) are, respectively:

$$\sigma_s/\pi a^2=(m-1)^2\cdot 2x^2=(1/2)\rho^2 \qquad (1<x<20)\,, \tag{3}$$

$$\sigma_s/\pi a^2=2-4\rho^{-1}\sin\rho+4\rho^{-2}(1-\cos\rho) \qquad (x\gtrsim 20)\,, \tag{4}$$
$$\rho=2x(m-1)\,.$$

In Fig. 1, the ratio of cross section of light scattering to geometrical cross area is drawn for ratio parameter $x\equiv(2\pi a/\lambda_0)$ by using equ. (1) over the range $x<20$ and equ. (2) over the range $x\gtrsim 20$. As numerically seen from equ. (4), the curve in this figure shows a maximum at wavelength λ_{max} where

$$4\pi a(m-1)/\lambda_{max}=2.08\,. \tag{5}$$

For obtaining accurate light scattering spectrum, transmission measurements at various wavelengths must be made under geometrical condition of narrow beam as mentioned, otherwise observed turbidity may become low owing to the light scattered into a small angle viewed off the direction of incident beam. With due consideration on this geometrical requirements, a single beam apparatus of indicating type was constructed as described in the preceding paper for studies on absolute values of light scattering cross section shown by spherical cells in suspensions. Since commercial spectrophotometers used in chemical studies are mostly designed for measurements of absorption in solutions or for purposes of practical turbidimetry in limited cases, they do not always meet the requirements for absolute turbidimetry. Therefore, a preliminary test was made on one of those spectrophotometers, Beckman Model DK-2, to see whether or not the type could be used for

recording scattering spectra of microbiol suspensions. Since forward scattering is of higher intensity for cells of larger diameter, *Saccharomyces cerevisiae* was used as a specimen of large diameter, probably the largest among the microbes to be studied.

In Fig. 2 were drawn curves obtained by plotting apparent turbidity τ' measured in absorbance scale in Beckman DK-2 against the population densities N of the specimen used. In the range not exceeding 0.8 in absorbance scale, a linear relation holds between τ' and N for every wave length used. The linear region, which will be called hereafter "dilute region", corresponds to the range over which the effect of multiple scattering can safely be disregarded so far as the data obtained by the use of this instrument is concerned. The absolute values of cross section of light scattering could, therefore, be found from inclinations of straight lines in Fig. 2. In Table 1, the values (σ_s^2) thus obtained are compared with those (σ_s^1) found by the use of standard narrow beam turbidimeter shown in a previous paper.

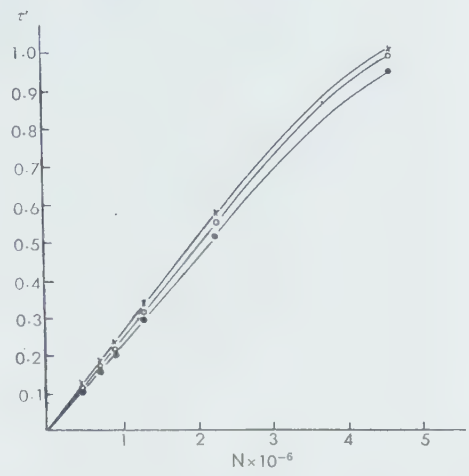


Fig. 2. Apparent turbidity vs. cell population.
× 504 mμ ○ 548 mμ ● 623 mμ

Table 1. Light scattering cross section at various wavelengths.

λ (mμ)	σ_s^1 (cm²)×10⁷	σ_s^2 (cm²)×10⁷
471	6.4	5.8
504	6.4	5.7
548	6.2	5.6
623	5.5	5.2

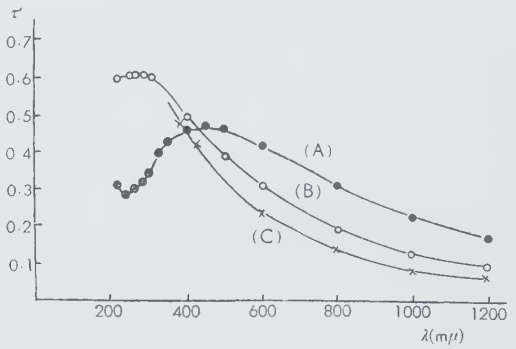
Although, owing to the lack of exact narrow beam condition, values of σ_s^2 were always somewhat smaller than the corresponding ones given in the row of σ_s^1, the difference did not exceed 10% of the original values at most. Moreover, differences of this sort are smaller for microbes of smaller diameter in accordance with the theory on angular distribution of scattered light. Commercially available spectrophotometer, Beckman Model DK-2, was therefore used as a working apparatus throughout the present work in which approximate patterns of whole spectra were more concerned than accurate values of light scattering intensity at any definite wave length.

GENERAL PROCEDURES OF LIGHT SCATTERING ANALYSIS

As theoretically expected of equs. (1) and (2), suspensions of cells of relatively large diameter might exhibit a maximum turbidity at a length in visible ranges. Examples of light scattering spectra are shown in Fig. 3 for spherical or quasi-spherical cells in dilute suspension. The observed average cells in dilute suspension. The observed average cell diameters $\langle D \rangle$ were 5.6 μ, 4.1 μ and 1.4 μ, respectively. From equ. (5), it can easily be proved that, at maximum wave length λ_{max}, there exists an approximate relation

$$\pi \langle D \rangle \Delta n / \lambda_{max} = 1.04 \qquad (6)$$

where $\Delta n \equiv n_1 - n_0$ and $\langle D \rangle$ indicates the average diameter of cells in suspension. If index increment Δn did not noticeably vary in its magnitude among the species used, the value of λ_{max} for smaller cells might be expected to shift to the shorter wavelength side as shown in Fig. 3.



(A) *Saccharomyces cerevisiae* (B) *Rhodotorula glutinis*
(C) *Micrococcus cancidus*

Fig. 3. Light scattering spectra.

In the following paragraphs, it will be shown that, by analyzing observed spectra of light scattering by means of the basic equations above described, one can evaluate in a perfectly spectrophotometrical manner the magnitude of cell diameter, cell index of refraction and population density of cellular suspensions.

(a) *Cellular Index of Refraction n*:

A series of sample suspension was made by suspending the same population of a test microbe in the same volume of media with various indicies of refraction (n_0). In preparing the media, precaution must be taken to maintain the osmotic pressure of the medium series at a costant level so as not to cause any noticeable osmotic changes in cell volume. For this purpose high polymer electrolytes such as salt-free water soluble proteins (e.g. serum albumin) were successfully used. Light scattering spectra were measured for each member of the suspension series, obtaining thereby a group

of curves similar in shape to curve (A), (B) or (C) in Fig. 3.

As in the range where $1<x<20$, cross section of light scattering, is given by equ. (3), the relative change in turbidity τ among the members of the series with varying index of refraction (n_0) is expressed as:

$$\tau = N\cdot\sigma_s = 2N\pi a^2(m-1)^2(2\pi a/\lambda_0)^2$$
$$= (1/2)N\pi^3\langle D\rangle^4(\Delta n/\lambda)^2 , \qquad (7)$$

where N is the population density and λ and λ_0 the wavelengths of the used light in vacuum and in a medium of refractive index n_0, respectively.

Consequently, if light of a proper wavelength (λ) is so chosen as to make ratio parameter (x) equal to or smaller than 20, the square root of the turbidity τ of the suspension series desceases in proportion to Δn. We can, therefore, find the value of cellular index of refraction n_1 as an intercept on x-axis by plotting $\sqrt{\tau}$ for n.

Fig. 4 shows two examples of evaluation of evaluation of n_1 by the use of this linear extrapolating method. The wave lengths used were 1 μ for yeast and 800 mμ for chlorella, respectively. Cellular indicies of refraction obtained in this manner give, therefore, the values at the prescribed wavelengths. In contrast to the interprolating method which usually necessitates the use of fairly large amount of a salt-free protein as described in a previous paper[1], the present one seems much more convenient in practical use.

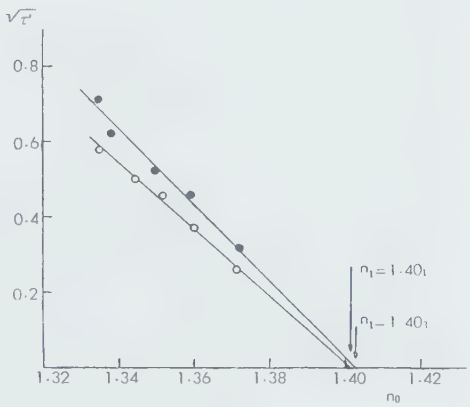


Fig. 4. Cellular indicies of refraction (n_1) as found by the extrapolating method.
○ Saccharomyces cerevisiae (1 μ)
● Chlorella ellipsoidea (800 mμ)

(b) *Average Cell Diameter* $\langle D\rangle$:

As numerically seen from equs. (1) and (2), the absolute value of $\tan\theta \equiv \dfrac{d[\log(\sigma_s/\pi a^2)]}{d[\log(1/\lambda_0)]}$ decreases monotonically as cell radius increases so far as ratio prameter x falls on the longer wavelength side of the spectral peak shown in Fig. 1. Average cells diameter $\langle D\rangle$ could, therefore, be obtained from the value of $|\tan\theta|$ at a properly chosen wavelength. Actually, the ratio of turbidities at two arbitrarily chosen wavelengths on an observed spectrum curve might provide a convenient measure to evaluate the value of $\langle D\rangle$.

Fig. 5 shows a calculated working curve for use in obtaining $\langle D\rangle$ from the observed values of τ at 800 mμ and 665 mμ. The curve corresponds to

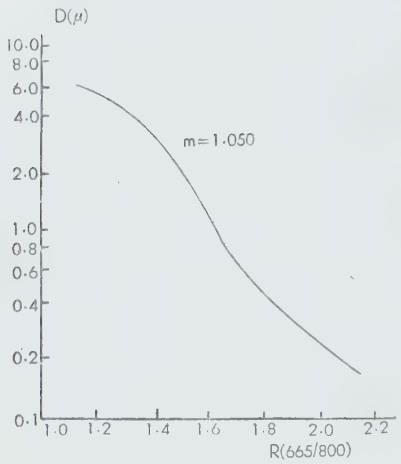


Fig. 5. Working curve for calculation of cell diameter

the case in which spherical cells of relative index of refraction $m=1.050$ are suspended in a medium of $n=1.333$. When $\langle D\rangle<2\mu$, the curve remains identical for all values of m, while if $\langle D\rangle\gtrsim 2\mu$, the curve takes different shape for different value of m necessitating the use of a particular curve computed by using the value of m observed as mentioned above.

(c) *Cell Population N*:

In dilute suspensions in which no multiple scattering occurs, the relation

$$\tau=N\cdot\sigma_s \qquad (8)$$

always holds, N being the cell number in unit volume. The value of τ could directly be observed and the use of m and $\langle D\rangle$ obtained as described in sections (a) and (b) respectively made it possible to compute therefrom the value of σ_s. The population desity (N) of spherical cells in suspension could thus be obtained in a perfectly spectrophotometrical manner. The values of N indirectly obtained agree fairly well with those given by counting under microscope.

SOME BIOLOGICAL APPLICATIONS

In the preceding sections, basic formulae and general procedures for the analysis of light scattering spectra of microbial suspensions were described. In the present section, some examples of the application to microbiological problems will be mentioned by using *Saccharomyces cerevisiae* as a test microbe.

(a) *Cell Volume Growth as Appeared in Spectrum of Light Scattering*

In Fig. 6 are shown a group of spectral curves given by a dilute suspensions of yeast cells when aerobically grown on a Knopps medium containing yeast extract. The culture showed no indication of population increase during the observation.

As easily seen from equ. (5),

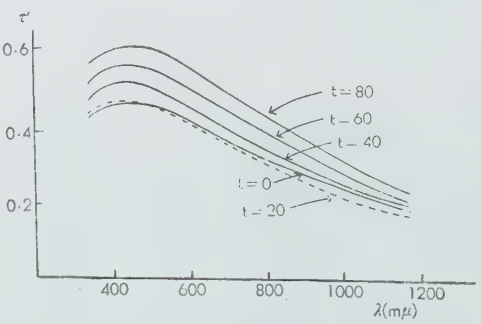


Fig. 6. Scattering spectrum change due to volume growth (*Saccharomyces cerevisiae*).

the first maximum on a turbidity-wavelength curve appears at a wavelength where $\lambda_0=(m-1)\pi\langle D\rangle/2.08$. At this particular wavelength

$$\rho\equiv 2x(m-1)=4.16\ , \qquad (9)$$

giving independently of m a universal value of 3.17 as the maximum value of $(\sigma_s/\pi a^2)$. Accordingly, if population density is known, cell radius (a) can be obtained from the maximum turvidity τ_{max} by using

$$\pi a^2=\tau_{max}/(3.17N)\ . \qquad (10)$$

As no population incraase was observed, the increase of τ'_{max} in Fig. 6 could be ascribed to the increase of cell diameter caused by incubation. The time rate of relative increase in cell diameter is shown as curve D in Fig. 7. Moreover, the peak wave-length in Fig. 6 remained practically unaltered during the growth period, indicating in accordance with equ. (6) that the cellular index of refraction (n_1) decreased as cell diameter $\langle D\rangle$ increased. In Fig. 7 the observed change of maximum turbidity is drawn together with computed curves for the time change of $\langle D\rangle$ and n_1. In this figure n_1 represents a value of cellular index for monochromatic light of wavelength λ_{max} which is 420 mμ in vacuum and 420 mμ/n_0 in a medium with index of refraction n_0.

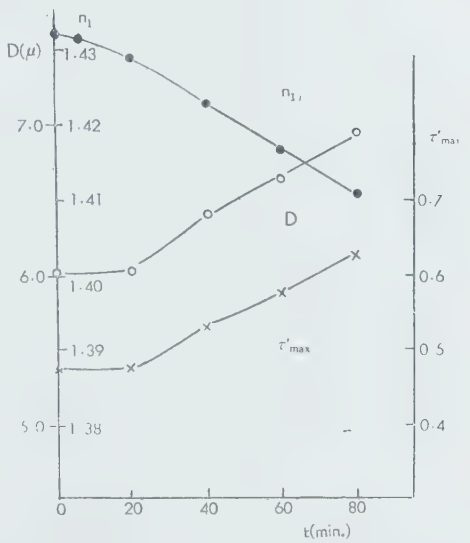


Fig. 7. Rate of change in cell parameters.

(b) *Osmosis as Appeared in Spectrum of Light Scattering*

If osmotic pressure difference is applied between extra-and intra-cellular media, it may possibly be balanced by the intrusion or extrusion of water passing through the semi-permeable cell membrane, causing, thereby, changes in both cell volume and cellular index of refraction. In general, turbidity cell suspension rose with the increase of cell volume and its index of refraction. If cells are suspended in a hypertonic medium, osmotic contraction occurs resulting in a decrease of cell volume and increase of refractive index, while if suspended in a hypotonic medium, osmotic expansion will be observed accompanied with an increase in cell volume and decrease in cell index of refraction. In both cases, an increase or a decrease might be observed in turbidity of the suspension, depending on which of the two opposing factors, volume effect and refractive index effect, is dominant.

For instance, as graphically seen in cells with relative index of refraction $m=1.05$ from Fig. 8 and could exactly be computed according to equ. (4), when a cell suspension was mixed with a hypertomic solution, the turbidity would increase owing to the index effect if the cells had a relatively small diameter, say 1 or 2μ, while it would decrease owing to the volume

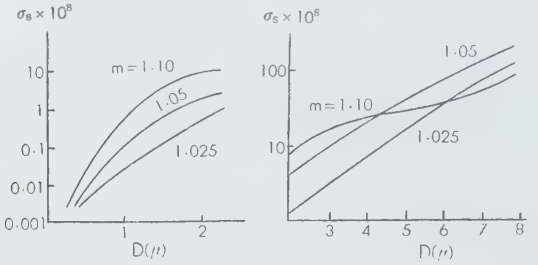


Fig. 8. Scattering cross section as functions of cell diameter (computed in accordance with equs. (1) and (2)).

effect if the cells were of a larger size, say 5 or 6 μ in diameter. The suspension of cells with medium size is, therefore, expected to exhibit practically no turbidity change because of the two opposing effects mentioned above.

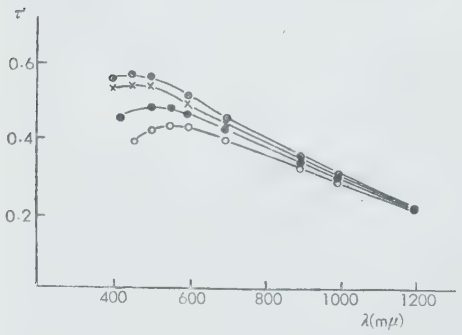


Fig. 9. Spectral changes caused by osmosis.
- ⊙ H_2O
- × 1×10^{-1} *M/l* (NaCl)
- ● 5×10^{-1} *M/l* (NaCl)
- ○ 1 *M/l* (NaCl)

In Fig. 9 is shown a group of spectra of *Saccharomyces cerevisiae* in NaCl solutions of various concentrations. It appears to be due to the valume effect that the turbidity decreases in shorter wave regions with the increase of NaCl concentration. If so, in longer wavelength side, the turbidity change must be observed in the oppsite direction. In Fig. 10, a difference spectra of the same suspension is drawn by using a suspension with equal population in pure water as a reference. At the longest side of the observed wavelength region, the curve is of negative sign as theoretically expected. If proper organism of smaller size were used, more prominent rise of turbidity might be realized by the osmotic contraction of cells in suspension. By considering the two opposing volume and index effects in the basic formulae for turbidity of cellular suspensions, the turbidity inversion of this kind could be explained physically so far as its main features are concerned without refrerring to any particular physiological mechanism.

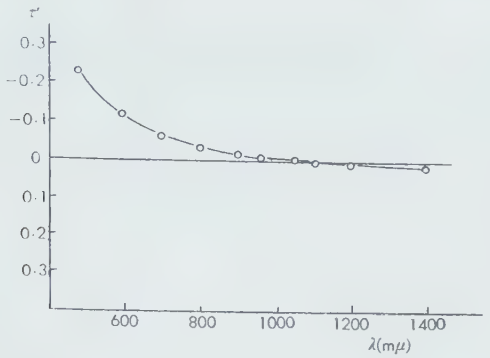


Fig. 10. Difference spectrum of yeast suspended in NaCl solution (referred to suspension in water).

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The authors are indebted to Dr. H. Iizuka of this institute for his kindness in supplying strains used in this study.

SUMMARY

By using monocellular microbes of spherical or quasi-spherical shape, spectrophotometrical studies on turbidity of cell suspension were made with the following results.

(1) Commercial spectrometers designed for solution chemistry can be used for approximate measurement of absolute turbidity of microbial suspension.

(2) Light scattering spectra of microbial suspensions could thereby be easily obtained making rossible "light scattering analysis" based on the Rayleigh-Gans' and Hart-Montroll's equations.

(3) General procedures for obtaining cellular index of refraction, cell volume and cell population were examined in the case of *Saccharomyces cereuisiae and Chrorella ellipsoidea.*

(4) "Light scattering analysis" gave some information on biological phenomena such as cell growth and osmosis in liquid culture.

(5) Variations of cellular index of refraction caused by volume change of cells explained the apparent inversion of turbidity change observed in osmosis of microbial suspension.

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PROTEIN SYNTHESIS IN *PSEUDOMONAS-P*

HIROMI MITSUI

Division of Enzymology, Institute of Applied Microbiology, University of Tokyo, Tokyo

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INTRODUCTION

During the past seven years, the number of investigations concerned with the mechanism of protein synthesis, both *in vivo* and *in vitro*, has been steadily increasing. The discovery of enzymes that activates the amino acid and transfer amino-acyl group to ribonucleic acid (*1*, *2*) has provided a new model for the intermediary biochemical events leading to the synthesis of protein. Out of the large body of information accumulated on this biochemical steps, has come the postulation of the following sequence.

Stage I. Activation of amino acids

AA+ATP+Enzyme=Enzyme-AMP-AA+PP

Stage II. Transfer of activated amino acids to carrier RNA

Enzyme-AMP-AA+s-RNA=s-RNA-AA+AMP+Enzyme

Stage III. Transfer to RNP, sequence determination, condensation and release of protein

$$\text{s-RNA-AA+RNP} \xrightarrow[\text{GTP}]{\text{Enzyme}} \text{peptide} \dashrightarrow \dashrightarrow \dashrightarrow \text{protein}$$

While this formulation was supported in many experiments in mammalian, plant and microbial systems, there are lots of findings which suggest alternate pathway leading to the synthesis of protein, for example, Beljanski's "amino acid incorporation enzyme" (*3*) and Sachs's "s" protein (*4*), and there are some possibility that structural protein of the ribosome themselves are made by a machanism different from that involved in the formation of soluble protein. Also the completion process of exo protein and proteins which are tightly bound to particulate system, for example α-amylase (*5*) and cytochromes (*6*), might be different from that of soluble protein.

Particularly in bacterial system, whether pure ribosome is the site of protein synthesis or ribosomes physiologically associated with the cell membrane are capable of completing the protein synthetic process is still obscure.

This article is an intermediary report of experiments which was attempted to shed a light on the above mentioned problem. A *Pseudomonas fluorescens* was chosen because this organism can synthesize a remarkable amount of soluble inducible enzyme pyrocatechase, and particulate bound protein, cytochrome c, under suitable conditions.

PHYSIOLOGICAL ASPECTS OF *PSEUDOMONAS-P*

A) *Microorganism*

This organism was isolated from soil for the study of the metabolism of pyrrolidone carboxylic acid (*7*). Taxonomical characteristics of the organism are as follows. Rod (0.6–0.8×1.2–1.8 micron), motile possessing one to three fragella and gram negative. This organism is classified as *psudomonas schuylkilliensis*. Table 1 summarizes the oxidative assimilation of various substrates by washed cell suspension. Mean generation time is 30 minutes

Table 1. QO_2 of washed cells for various substrates.

Substrates	Glutamate	α-ketoglutarate	Glucose	Succinate	acetate
3 hr. cells	110	10	20	70	20
8 hr. cells	54	15	60	50	110

QO_2 are expressed as microliters of oxygen taken up per hour per mg. of dry weight of cell.

Composition of reaction mixture: Cell suspension 0.5 ml. 0.2 M phosphate buffer (PH 7.0) 0.5 ml., 0.1 M substrate 0.1 ml.

in pepton-bouillon medium and 40 minutes in glycerol-glutamate or succinate medium at 30° with aeration.

B) *Inducible pyrocatechase*

1) The relationship between growth phase and induced synthesis of pyrocatechase

It will be seen in Fig. 1 that the ability of cells for the induced synthesis of pyrocatechase changes from time to time on the course of growth, and the cells in stationary phase showed optimal activity. At twenty minutes after induction the net synthesis of enzyme starts and the rate of enzyme synthesis reaches its maximum at forty minutes and afterwards it decreases gradually and ceases after eighty minutes.

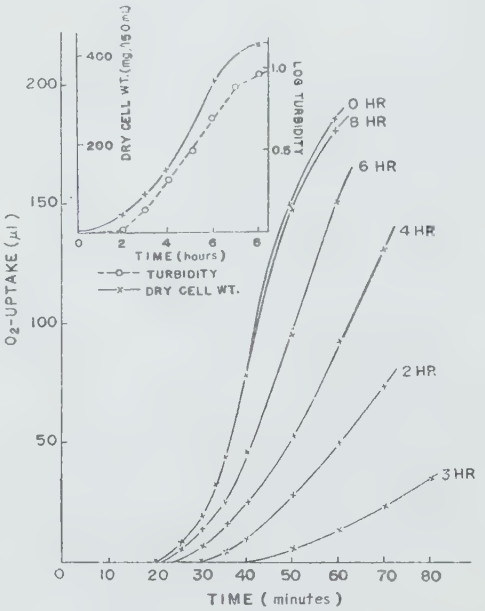

Fig. 1. Relationship between growth phase and pyrocatechase adaptability.* Composition of reaction mixture of oxidation: cell suspension 0.5 ml., *M*/5 phosphate buffer (pH 7.0) 0.5 ml., *M*/100 catechol 0.2 ml. Data are expressed as microliters of oxygen taken up per mg. of dry weight of cells and corrected for endogenous respiration. The insert is the growth curve.

* Cells were collected at various time by centrifugation washed and resuspended in water. Inducible formation of pyrocatechase in resting cells was followed by the oxidation of catechol.

2) Purification of pyrocatechase and some properties of enzyme

The procedure of purification of the enzyme is similar to that described by TOKUYAMA (*8*). The organism were grown at 30° aerobically in pepton-bouillon medium containing 0.2 per cent benzoic acid as inducer. After sixteen hours, cells were harvested, washed and sonicated. 16,000×g. supernatant of sonicate was mixted with one tenth volume of 10 per cent streptomycin solution to remove nucleic acid containing materials. Ammonium sulfate fractionation collecting the precipitate between 35 and 45 per cent saturation repeated three times. After negative adsorption on calcium phosphate gel, enzyme sample was applied on starch column for zone electrophoresis. Enzyme preparation thus purified, have specific activity about 3900 μl. O_2 uptake/hr/mg. protein. This enzyme needs ferrous ion for its full activity and stabilization of enzyme in manometric assay. So, the composition of reaction mixture

is 0.1 ml. enzyme solution, and 0.9 ml. of $M/5$ phosphate buffer (pH 7.0) in main Warburg flask and 0.1 ml. of 2×10^{-3} M ferrous sulfate dissolved in 0.1 per cent of ascorbic acid, 0.1 ml. of 0.1 M catechol and 0.3 ml. of $M/15$ phosphate buffer in side flask. For the assay of purified enzyme, spectrophotometry is useful as reaction product, cis-cis muconic acid, has strong absorption at 257 mμ and in this case there is no need of ferrous ion. Tokuyama (*8*) isolated pyrocatechase of *Micrococcus urea* in zone electrophoretically pure form and its specific activity was 42,000 μl O_2 uptake/hr/mg. protein and the homogenity of this sample was confirmed from its analytical centrifuge pattern, and molecular weight of this enzyme estimated approximately 84,000.

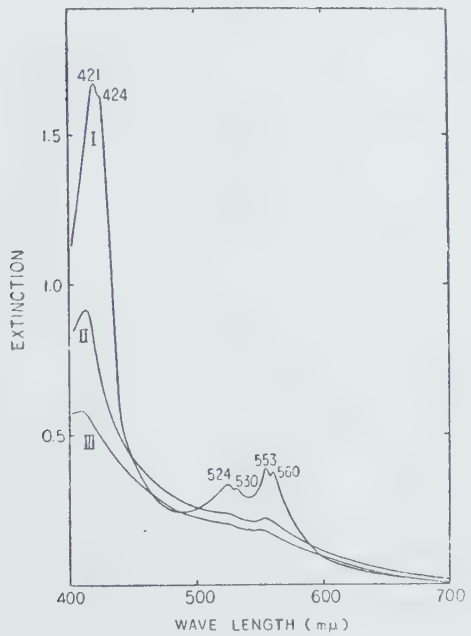

Fig. 2. Visible absorption spectra of the cells grown in different media. Each growth was carried out by shaking at 30° in 100 ml. of medium in Sakaguchi flask after 1% inoculation. I, III and II are the spectra for the cell suspensions at a concentration of 100 mg. of wet cells per 1 ml. of the suspension, which have grown for 16 hours in bouillon-peptone, casamino acid-glucose and casmino acid-glucose with the addition of 2 mg. iron (as ferric ammonium citrate) respectively.

C) *Cytochrome c*

1) Cytochromes of *Pseudomonas-P*

A typical absorption spectrum of *Pseudomonas-P* cell suspension is shown in curve I of Fig. 2. As the curve shows, *Pseudomonas-P* has the absorption maximum at 421, 424, 524, 530, 553 and 560 mμ within the range from 400 to 700 mμ. This finding seems to indicate that *Pseudomonas-P* has c-like (553 mμ as α band) and b-like (560 mμ as α band), but no a-like cytochrome, the absorption maximum of which lies at the range from 560 to 700 mμ. In other words, this microorganism has the ability to synthesize cytochromes 553 and 560, if certain growth condition is given.

The spectrum of the living cell suspension was almost the same as that of the disrupted cell suspension to which a few crystals of sodium dithionite were added. This finding seems to show that both cytochromes 553 and 560 are present almost in the reduced form at the stationary state of living cells.

2) The variation of cytochromes in response to that of the growth medium or the growth phase

The absorption spectrum of *Pseudomonas-P* cell suspension is not always similar to curve I of Fig. 2. Curve I of Fig. 2 shows the spectrum of the cells cultivated for 16 hours in peptone-bouillon medium. Curve III and II show the spectra of the cell cultivated for 15 hours in iron-poor and -rich casami no acid-glucose media, respectively. The growth rate was almost similar in three media. The results seem to indicate that cytochrome content varies in response to nutritional conditions.

The variation of cytochromes were also observed in response to different growth phase. Fig. 3 shows the absorption spectra, measured by the opal

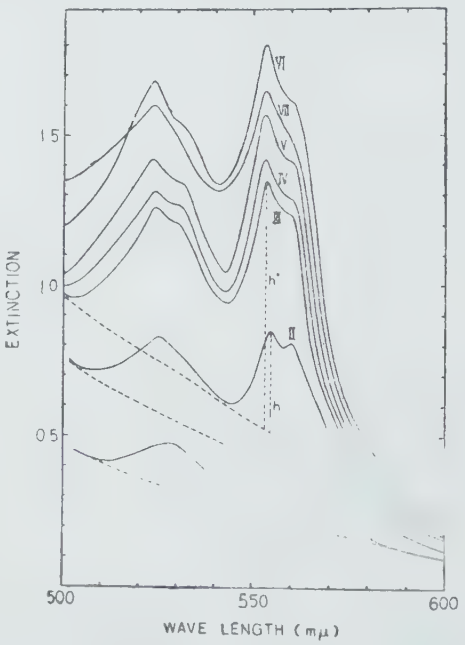

Fig. 3. Visible absorption spectra of the cells at different growth phases. The cell growth was carried out by shaking at 30° in bouillon-peptone medium in Sakaguchi flask after 1% inoculation. Spectra correspond to cell suspensions containing 100 mg. of cells (dry weight) per ml. of suspension. I, II, III, IV, V, VI and VII are the spectra for the cells grown for 3, 4, 5, 6, 7, 10 and 14, and 24 hours, respectively.

glass method, on the washed cell suspensions at different growth phase. Fig. 4 shows the relationship between growth and cytochrome content, which is expressed tentatively by the strength of the maximum absorption at the range from 550 to 560 mμ. As will be seen in Fig. 3-I, the cells incubated for 3 hours had only little cytochromes and had more 560 component than 553 component. As the growth proceeded, cytochrome content of the cells increased remarkably and the formation of cytochrome 553 surpassed that of cytochrome 560. When the cell growth reached the stationary phase, the increase of cytochrome content stopped and the spectrum of the cell suspension hardly varied thereafter.

3) The distribution of cytochromes on the cellular components of *Pseudomonas-P*

The cells at different growth phase were disrupted by sonic oscillation and fractionated by differential centrifugation, (scheme 1). The suspension

in 0.1 *M* sodium phosphate buffer pH 7.0 of each fraction was measured for its absorption spectrum by the opal glass method in the presence of few crystals of sodium dithionite. The content of cytochromes in each fraction is shown in Table 2. No qualitative difference was observed, namely the

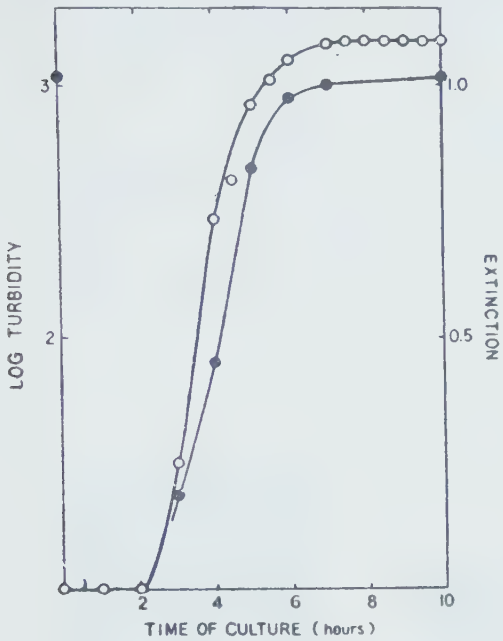

Fig. 4. The relationship between the cell growth and the cytochrome content. -○- turbidity in logarithms -●- cytochromes content corrected on the basis of the same dry weight of cells). Cytochrome content is expressed tentatively with the extinction h, h′, h″ etc. on Fig. 3 (the maximum extinction within the range from 550 to 560 mμ).

Table 2. The distribution of cytochromes and nitrogen on the fractions of sonicated cells.

The culture was performed by shaking at 30° for different time intervals on bouillon-peptone medium in Sakaguchi flask after 1% inoculation.

Time of culture	Fraction	LP	SP	S
3 hours	nitrogen[1)]	0.041	0.196	0.992
	cytochromes[2)]	0.000	0.025	0.015
	cyt./mg N	0	0.13	0.015
7 hours	nitrogen[1)]	0.145	0.604	1.450
	cytochromes[2)]	0.105	0.850	0.275
	cyt./mg N	0.72	1.40	0.19
24 hours	nitrogen[1)]	0.176	0.694	2.100
	cytochromes[2)]	0.170	0.860	0.750
	cyt./mg N	0.97	1.24	0.36

1) Milligram nitrogen per ml. of the suspension.

2) The value is expressed as the maximum extinction of the suspension at the range from 550 to 560 mμ.

ratio of cytochrome 553 to 560 was almost similar in every fraction. However, a significant difference in quantity of cytochromes was observed, and sp fraction contains more cytochromes than other fractions. Moreover, the difference was most evident in the cells cultivated for 7 hours and less evident in the cells cultivated for 24 hours. This finding suggest that both of cytochrome 553 and 560 are localized on a particle which belongs to sp fraction, and that such structure might become fragile to mechanical disruption in late stationary phase.

4) The extraction and the purification of cytochromes

Cytochrome 560 was more difficult to extract than 553 component. So the purification was restricted only to cytochrome 553. Extracted cytochrome 553 was partially purified by ammonium sulfate fractionation (between 0.2 and 0.5 saturation) and column chromatography on aluminium oxide bufferized with 0.05 *M* sodium phosphate buffer, pH 7.0. The absorption spectrum of partially purified cytochrome 553, which was measured without opal glass, showed that this pigment has the absorption maxima at 551, 523 and 419 mμ in the reduced form, giving a ratio 9:1 in the absorption at 280 mμ to that 551–552 mμ (Fig. 5). This pigment is not autoxidizable. The slight difference between the spectrum of the purified pigment and that of living cells seems to be due to the overlapping of the absorption of 553 and 560 components in the living cell or else the difference in the method of spectrophotometry.

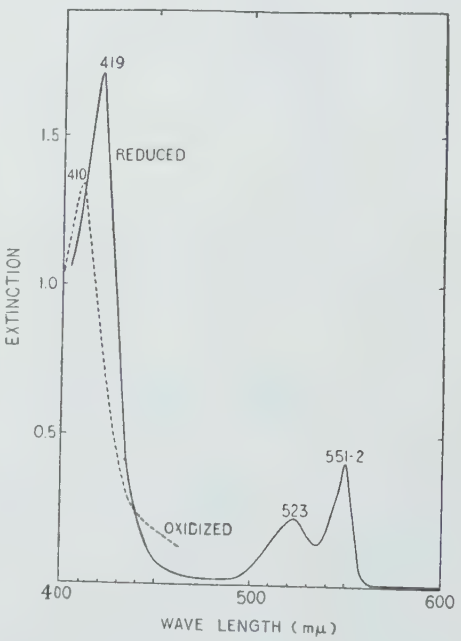

Fig. 5. The absorption spectrum of partially purified cytochrome C_{553}.

PHYSICAL AND CHEMICAL PROPERTIES OF CELL FRACTIONS

A) *Fractions from sonically disruped cells*

Procedure for the disruption of the cells and fractionation is summarized in scheme 1.

S_2 fraction was analysed with Spinco Model-E ultracentrifuge and Spinco Type-H Tisselius electrophoresis assembly equipped with optical system and all-quartz cells.

Peak I corresponds to the sp fraction. Peak II and peak III are the main components of the s fraction.

Table 3 summarizes the distribution of protein and ribonucleic acid in the three fractions. The Lp fraction was low in RNA content and contained

Scheme 1. Preparation of Sonic Extract.

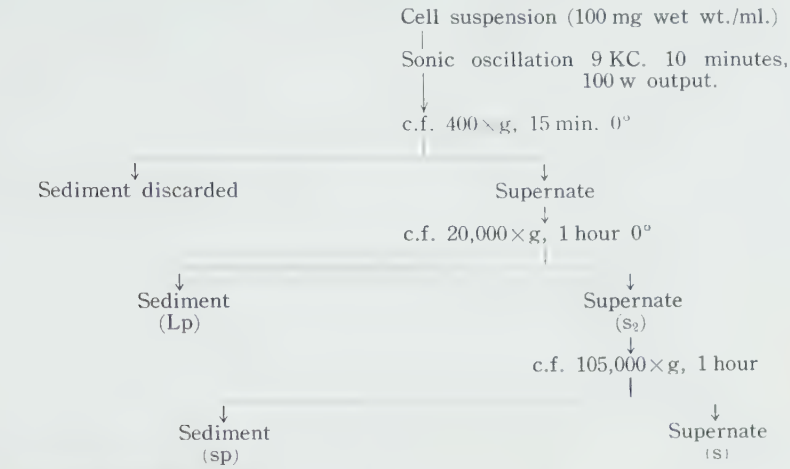

Photo. 1. Ultracentrifuge pattern of S_2 fraction.

Photograph was taken after the sedimentation for about 20 minutes at 20° and 39,460 r.p.m.

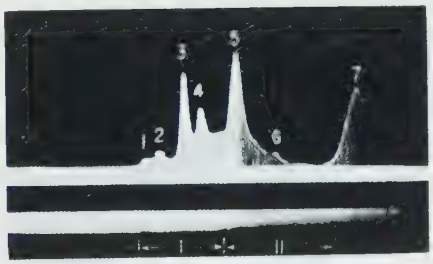

Photo. 2. Electrophoretic Patterns of s_2.

Table 3. Distribution of RNA and protein in fraction.

Fraction	per cent of total	
	RNA	protein
Lp	2.1	6.0
sp	28.1	16.0
s	69.6	78.0

much lipid material. 30 per cent of RNA and 70 per cent of cytochromes associated with sp fraction. More than 70 per cent of RNA and portein come into s fraction. Photo. 2 is the illustrative electropheoretic pattern of s_2, showing that at least seven components are present. Absorption spectra of samples taken from section I and II are shown in Fig. 6 a, b. In the

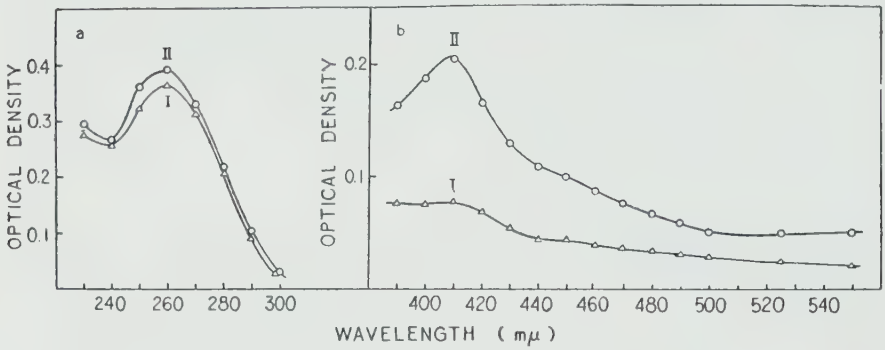

Fig. 6. Absorption spectra of samples taken from section I and II.

ultraviolet region from 230 to 300 mμ, the absorption spectra of both samples gave simillar patterns qualitatively, and quantitatively indicating the content of RNA were the same in both sections. In the visible region from 390 to 550 mμ, only the samples from section II showed strong absorption at 420 mμ, indicating the presence of cytochromes.

The boundary electrophoretic patterns of Lp and sp and s are shown in Photo. 3 a, b, c respectively. Lp showed a single peak, sp was composed

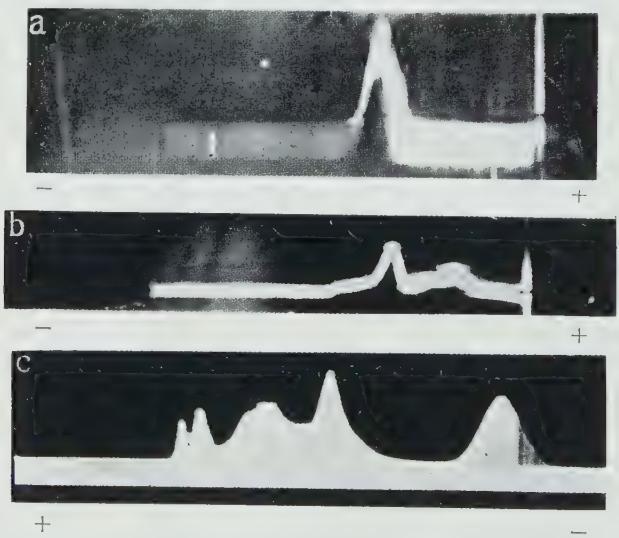

Photo. 3. Boundary electrophoretic patterns of Lp, Sp and s. (Ascending limb, tris-HCl buffer, ionic strength 0.01, pH 8.35).

of three main components and in s, at least six components were distinguished. The characteristics of each components were further investigated on starch column electrophoresis. As shown in Fig. 10a s_2 separated into four distinct protein peaks (1, 2, 3, 4) and two RNA peaks (I, II). Peak II

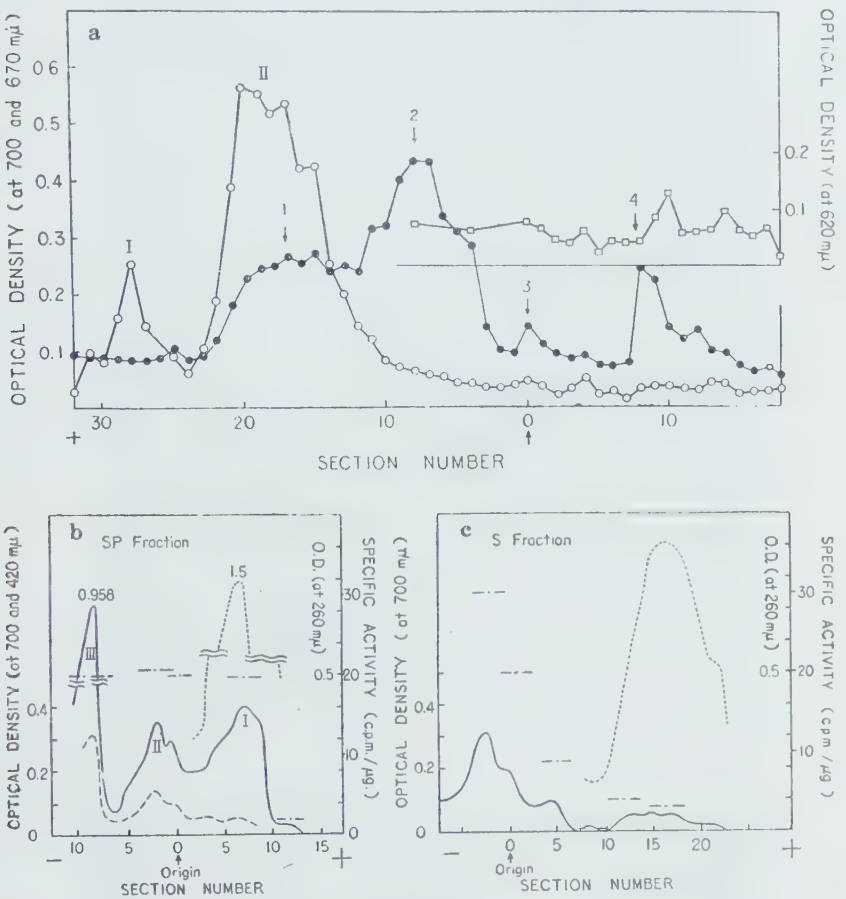

Fig. 7. Zone electrophoretic patterns of s_2, sp and s.

—●—: Protein (700 mμ). ·····: Nucleic acid (260 mμ).
-○-: RNA (670 mμ). - - - -: Cytochrome (420 mμ).
—□—: Polysaccharide (a20 mμ). — · —: Specific activity (cpm/μg).

was accompanied by protein, and from its RNA and protein content, this component might be a ribonucreoprotein particle. The sp fraction separated into a ribonucleoprotein particle and two cytochrome containing particles (Fig. 7 b). S fraction was separated roughly into a protein component and RNA rich component.

B) *Fractions from osmotically disrupted protoplasts*

Scheme 2 shows the procedure for preparation of protoplast, disruption and centrifugal fractionation. Protein and ribonucleic acid were distributed to both fractions, p and s, in nearly equal amount (Table 4).

Scheme 2. Procedure for the fractionation of disrupted protoplasts.

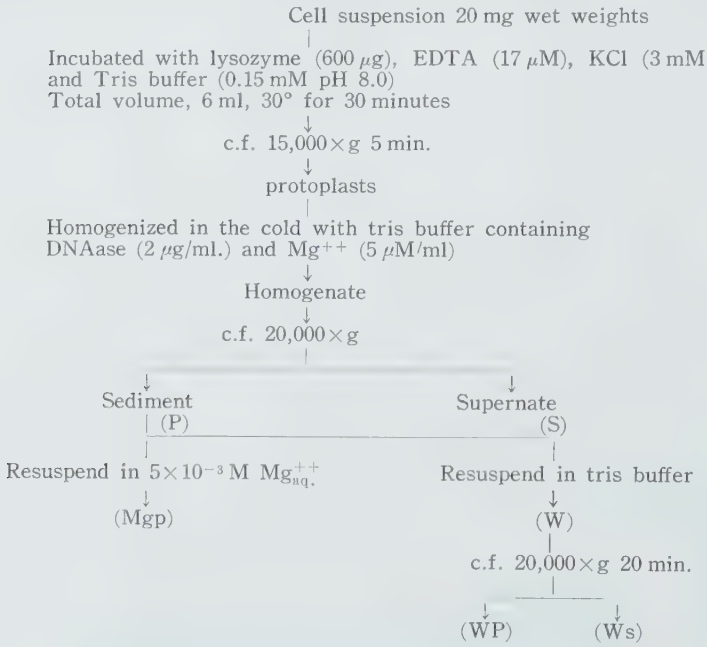
Cell suspension 20 mg wet weights
Incubated with lysozyme (600 μg), EDTA (17 μM), KCl (3 mM) and Tris buffer (0.15 mM pH 8.0) Total volume, 6 ml, 30° for 30 minutes
c.f. 15,000×g 5 min.
protoplasts
Homogenized in the cold with tris buffer containing DNAase (2 μg/ml.) and Mg++ (5 μM/ml)
Homogenate
c.f. 20,000×g
Sediment (P)
Supernate (S)
Resuspend in 5×10⁻³ M Mg++aq.
(Mgp)
Resuspend in tris buffer
(W)
c.f. 20,000×g 20 min.
(WP)
(Ws)

Table 4. Distribution of RNA and protein in p and s fractions.

Fraction	Per cent of total	
	RNA	Protein
p	42.5	51.8
s	57.5	48.2

Photo. 4 a, b, c are the electron micrograph of Mgp, Wp, and intact cell. Compared to Mgp, Wp is more transparent against the electron beam. This might be due to a release of macromolecular components from p to supernatant fraction, WS, in the low concentration of magnesium ion. Chemical analysis showed that more than 60 per cent of protein and 75 per cent of RNA was released into supernatant by washing once with tris buffer containing magnesium lower than 10^{-4} *M*. Washing with buffer containing magnesium over 5×10^{-3} *M* gave no release of protein and RNA (Table 5). Analytical centrifuge pattern showed that released material from p consisting of 56s, 46s, and 31s particles and materials lower than 4s (Photo. 5).

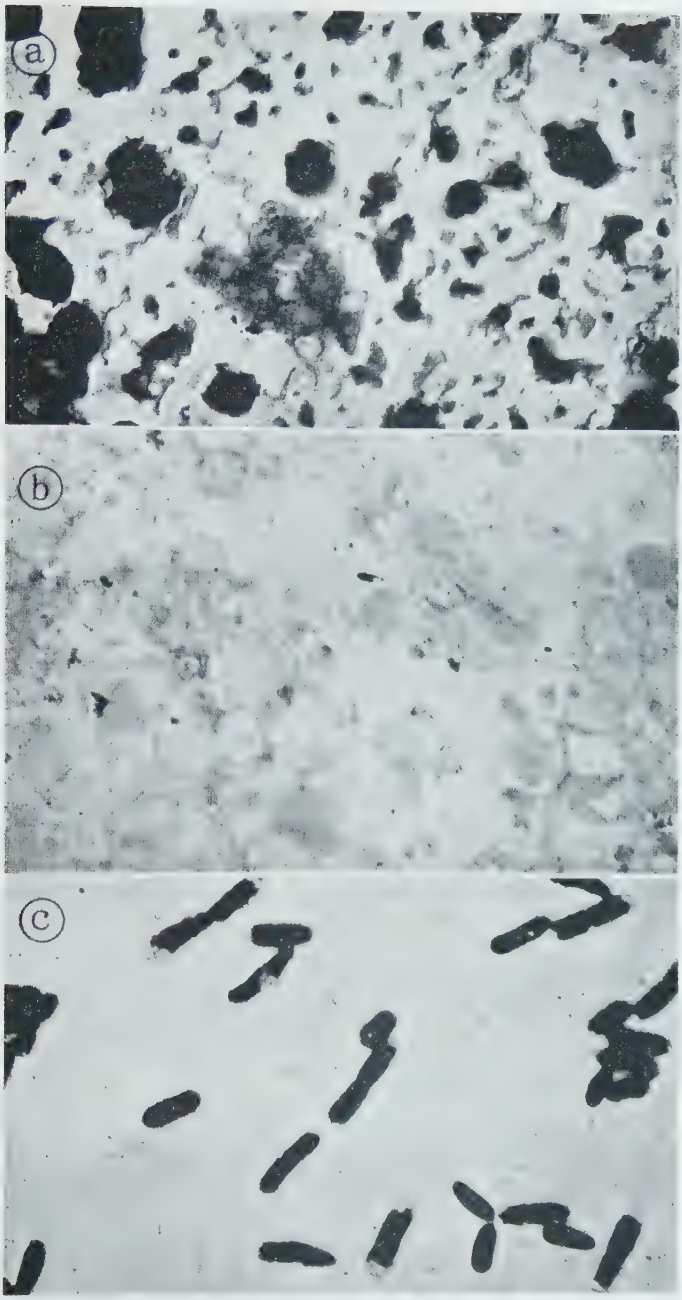

Photo. 4. Electron micrography of subcellular fractions. (a) MgP fraction, ×5750, (b) WP fraction, ×5750, (c) Intact cells of *Pseudomonas P*, ×5750.

Table 5. Analysis of released components.

Component \ Fractions	MgP	WP	WS
Protein[1] (μg. tyrosine equiv.)	220	85	130
RNA[1] (μg)	770	170	600
Particles[2]	—	—	56s, 46s, — 31s
Amino Acid activating enzymes[3] (POP μM exchanged/10 min.)	0.75	0.06	0.90

1) One ml. of each fraction was analysed according to the methods described in the previous section.

2) Sedimentation analysis was carried out with Spinco ultracentrifuge model E.

3) Each flask contained 0.1 ml. of preparation, 1 mg. of casamino acid, 5 μM of ATP, 5 μM of P^{32}-pyrophosphate (30,000 c.p.m./μM) 5 μM of $MgCl_2$ and 100 μM of tris buffer in 0.5 ml. Reaction mixture was incubated at 30° for 10 minutes.

These particles are similar to so-called ribosome in their ultracentrifuge patterns and protein RNA ratio.

From these results, it could be concluded that p or Mgp is cell membrane fraction which is associated with ribosome-like particles.

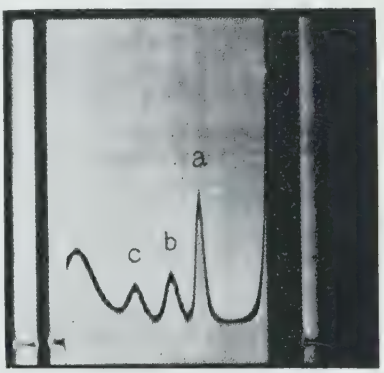

Photo 5. Sedimentation pattern of WS fraction.

Patterns were recorded 82 minutes after attainment of full speed, 20,410 r.p.m. Sedimentation coefficients were (a) 56s, (b) 46s, (c) 31s.

INCORPORATION OF AMINO ACIDS INTO PROTEINS OF CELL FRACTIONS *IN VIVO*

A) *Distribution of radioactivity on sonically disrupted cell fractions*

Intact cells were exposed to labeled amino acids for 2 minutes. Protein synthesis being stopped by chilling and the use of 0.1 *M* sodium cyanide, cells were freed from reaction medium by washing twice at 0°C, and disrupted sonically. Cell homogenate was fractionated as described in scheme 1.

Results are summerized in Table 6. The radioactivity incorporated in s was nearly equal to the sum of that of particles. However, the specific activities of the particulate fractions were seven times higher than that of s. Further zone electrophoretic analysis of each fraction showed that specific

Table 6. Incorporation of amino acids into protein of pseudomonad cell fractions *in vivo*.

Fraction	Total proten (μg of tyrosine equivalent)	Total activity c.p.m.	Specific activity (c.p.m./μg tyrosin equivalent
Lp	610	15,800	26
sp	440	9,700	23
s	6,120	26,100	4.3

Five ml. of cell suspension (500 mg. in wet weight) was incubated with ^{14}C-amino acid mixture (500,000 c.p.m.) in *M*/15 phosphate buffer and 10 mg of casamino acid.

radioactivities of three components of sp were nearly the same and in s, labeling was inhomogeneous and a soluble protein fraction labeled preferentially (Figs. 7 b and c).

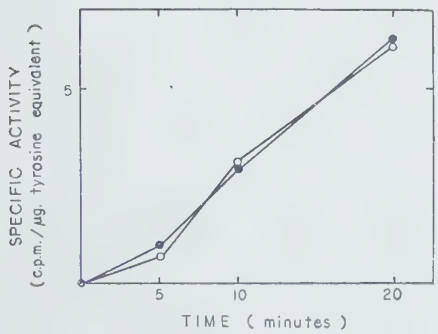

Fig. 8. Amino acid incorporation into p and s fraction *in vivo*. 50 mg of cells were suspended in 8.8 ml. of *M*/10 phosphate buffer (pH 7.3) and 1.0 ml of 10 per cent casamino acid and inqubated aerobically at 30°C. After temperature equilibrium, 0.2 ml. of ^{14}C-amino acid mixture (200,000 c.p.m.) was added. ○—S, ●—P.

B) *Distribution of radioactivity in membrane fraction and cytoplasmic supernate*

Cells exposed to labeled amino acids were disrupted via protoplasts. Results of the experiment are shown in Fig. 8. The specific activities of P and S increased linearly during the course of experiment for 20 minutes, and rate of labeling of each fraction was nearly equal.

AMINO ACID INCORPORATION INTO ISOLATED FRACTIONS

A) *Fractions from sonicate*

In Table 7 the results of the experiments with Lp, sp and s are summarized. The abilities of Lp and sp to incorporate amino acids were much higher than the ability of s. S showed a marked inhibitory effect on the incorporation of amino acid by particles. The activities of these particles *in vitro* are very weak compared to that *in vivo*. Sonic disintegration of *Pseudomonas* cells may be too drastic to prepare active fractions.

B) *Membrane fractions and cytoplasmic supernate.*

Homogenate obtained by disruption of protoplasts incorporates amino acids linearly for more than 30 minutes at the rate of 1/3 of that of the intact cells. At various times reaction was stopped by sodium cyanide and chilling, and homogenate separated to P and S fractions. Both fractions were

Table 7. Incorporation of ^{14}C-amino acids into isolated fractions.

	Lp	Lp+s[1)]	sp	sp+s	s
Radioactivity c.p,m./100 μg tyrosine equivalent	65	27	42	5.0	4.0

Reaction condition: 30°, aerobic for 60 min.
Reaction mixture: 0.5 M phosphate buffer (pH 7.4), 0.2 ml. 10 per cent casmino acid, 0.1 ml. 180,000 c.p.m. ^{14}C-amino acids mixture, 0,2 ml. 0.5 mg. protein for sp and 1.1 mg protein for s. Total volume 1.8 ml.

1) Particular fraction was separated from soluble fraction after the incubation and specific activity estimated as to Lp and sp only.

labeled as shown in Fig. 9. The viable cell count of homogenate was less than 10^6 and no intact cell was detected by both optical and electron microscopic observations. The S or the P fraction resuspended in buffer (W) did not incorporate amino acids into the protein fraction by itself (Fig. 9). Though the addition of the S fraction to the W did not restore the activity, the P fraction resuspended in the S fraction showed nearly the same activity of that of the P fraction in homogenate (Fig. 9). It was found that magnesium ion present in the s fraction at the concentration of $5\times10^{-3}\,M$ was essential as a component of resuspending medium to keep the P fraction active. The P fraction resuspended in magnesium of appropriate concentration completely restored its activity, but the addition of magnesium ion to the W did not restore its activity. Optimal concentration of magnesium ion is $10^{-2}\,M$ and at concentration less than $10^{-4}\,M$ more than 95 per cent of activity was lost (Fig. 10). It was described previously that ribosome-like particles were released from P into the S fraction when the P fraction was resuspended in buffer containing magnesium lower than $10^{-4}\,M$. The amino acid dependent

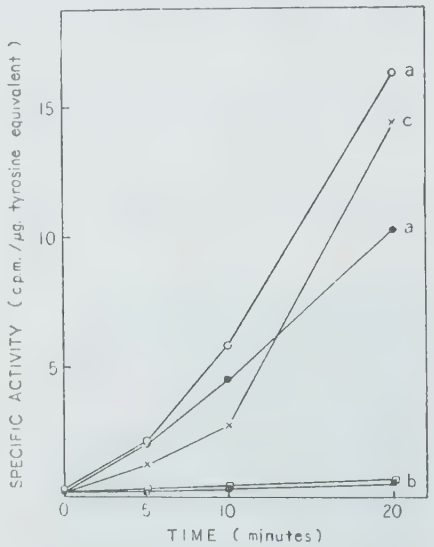

Fig. 9. Amino acid incorporation by isolated components.
(a) The homogenate was incubated with C^{14}-amino acid mixture and separated by subsequent centrifugation to precipitate and supernate. -●- P, -○- S.
(b) The W fraction and the S fraction was respectively incubated with C^{14}-amino acid mixture. -□- W, -■- S.
(c) The P fraction resuspended in the S fraction was incubated with C^{14}-amino acid mixture. -×-
Each flask contained 1 ml. of subcellular fraction, 1 mg of casamino acids, C^{14}-amino acid mixture (40,000 c.p.m.) and 2.4 ml. of $M/5$ phosphate buffer. The final volume was 4.0 ml.

pyrophosphate-ATP exchange activity was also released into Ws fraction completely (Table 4). It can be concluded, therefor, that 10^{-2} *M* to 10^{-3} *M* of magnesium ion in the suspending medium was essential to maintain the integrity of the P fraction. It is worth noticing that labeling of soluble protein only proceeds in the combined system of P and S.

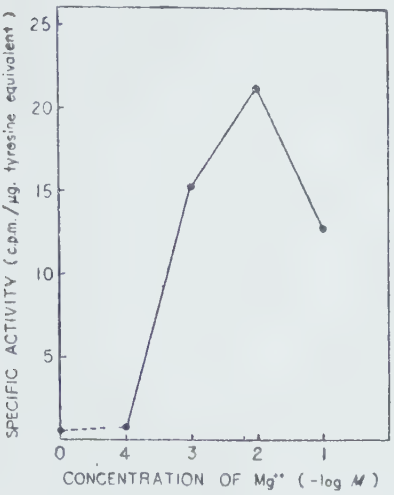

Fig. 10. Effect of concentration of magnesium ion in resuspending medium on the activity.

Each flask contained 1 ml. of preparation, resuspended in various concentrations of $MgSO_4$, 1 mg. of casamino acid, C^{14}-amino acid mixture (60,000 c.p.m.), 0.9 ml. of *M*/5 phosphate buffer (pH 7.2). Final volume was 4.0 ml. Incubation was continued for 30 minutes.

AMINO ACID INCORPORATIONS INTO SPECIFIC PROTEIN *IN VITRO*

The membrane fraction incorporates amino acid into protein very actively (1/3 of intact cell) and one can expect that the net protein synthesis might occure in this system. In order to determine this possibility, the isolation of a well characterized radioactive protein from this system would be required. Experiments for the synthesis of pyrocatechase was postponded because inducer, catechol, itself is a strong inhibitor for amino acid incorporation in this system even at the concentration of 10^{-4} *M*.

Cytochrome c coherent to Mgp was chosen as a specific protein. It will be seen in Fig. 11, incorporation of radioactive amino acids into protein by Mgp proceeds continuously for 60 minutes. At 60 minutes, reaction mixture was poured into cold aceton (−20°C) and stirred for 30 minutes to remove lipid, and then the precipitate was collected by centrifugation. Cytochromes were extracted with sodium citrate and impurities was removed by negative adsorption on XE-64 cation exchanger and ammonium sulfate fractionation. Precipitate (between 35 and 95 saturation) was chromatographed on aluminium oxide column. The coincidence of radioactivity with cytochrome 554 peak (Fig. 12) demonstrated that ^{14}C-labeled cytochrome c_{554} has been formed. Radioactivity recovered in cytochrome c_{554} fraction amounted to approximately 5 per cent of that incorporated into protein of Mgp.

DISCUSSION

It was demonstrated that, thus far, the most active preparation for the synthesis of protein obtainable from *Pseudomanas-P* have been associated

with cell membrane, and these cell membrane preparation sediment at force close to those which sediment whole bacterial cells. ROGERS and NOVELLI (*9*) reported that the efficiency of protein synthesizing ability of cells is increased several fold by the addition of bacterial extracts. Recently LAMBORG and ZAMECNIK (*10*) found that in *E. coli* cell-free system, when the number of viable count is 1×10^5 per ml. or less, the amount of labeled protein is not above background and over 1×10^6, labeling of protein due to viable cells increase in proportion to the number of cells. This danger of contamination of subcellular fractions by whole bacteria was eliminated to some extent in this experiment. Viable cell counts is lower than 10^6/ml. and whole cell was not found under optical and electron microscopic observation and, further, magnesium effect on the integrity of membrane fraction might serve as better evidence for this question.

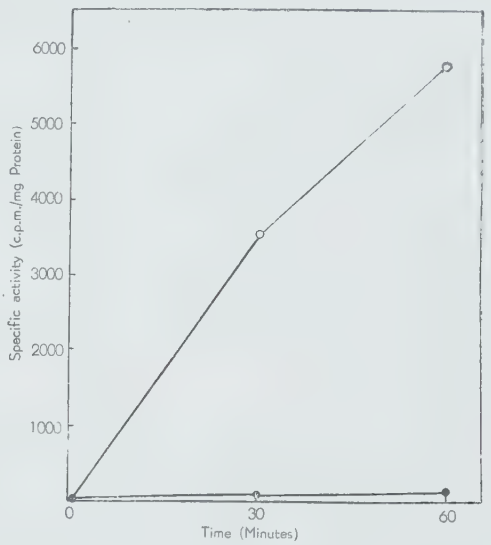

Fig. 11. Amino acid incorporation into whole protein of MgP and WP.

Reaction mixture contained 11.5 mg. (protein) of preparation, C^{14} amino acid mixture (3×10^5 c.p.m.), 5 mg. of peptone and 0.2 m*M* of phosphate buffer (pH 7.2). Final volume was 15 ml. Incubation was carried out aerobically at 30°.
○———— Mgp, ●———— WP

Several workers (*2, 11–16*) have attempted to fractionate various disrupted bacterial cell preparations and they claimed that the unequivocal protein synthesis seems to be more closely associated with the fragmented cell membrane material than with the ribosomes or the other less easily sedimentable fractions of the cells. On the other hand, it was claimed that $100{,}000\times$g (2 hr) pellet of *E. coli* extract obtained by alumina-grinding actively incorporates C^{14}-amino acids into protein and this activity is closely dependent on ribosomes, soluble enzymes ATP, GTP and an amino acid mixture (*10*). Our experiments partly dissolved this apparent discrepancies. Membrane preparation actually contains ribosome-like particles and amino acid activation enzymes and these components were released from membrane by lowering the magnesium ion concentration below 1×10^{-4} *M*. Very recently HUNTER et al (*17*) also reported the release of particulates from *B. megatherium* membrane preparation by washing with low concentration of phosphate buffer. If the 70 s ribosome is the actual site of protein synthesis, the ability

of amino acid incorporation in W preparation should be restored by the ajustment of magnesium ion concentration to 5×10^{-3} *M*. But this is not the

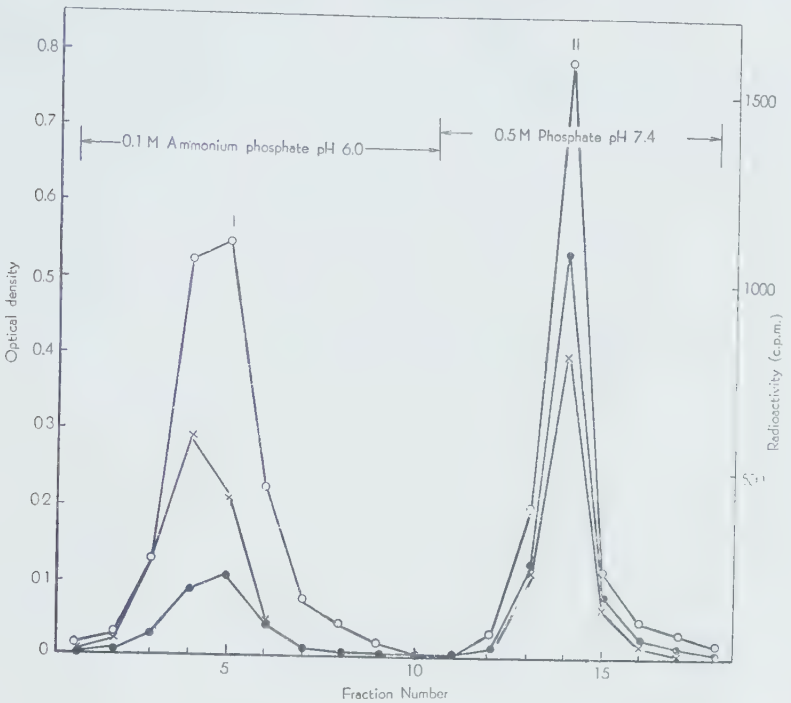

Fig. 12. Chromatographic patterns of radioactivity, protein concentration and 413 mμ absorption.

○———— Protein (Folin-phenol. 700 mμ absorption).

●———— 419 mμ absorption.

×———— radioactivity (c.p.m.).

case. In the present stage, it is not clear whether ribosomes released from membrane preparation by this method are affected by some kind of denaturation. One might be able to imagine the role of bacterial membrane as something like endoplasmic reticulum of mamalian cells (*18*). But, as could be seen in section V-B, membrane fraction itself synthesizes its own bound protein and, only in the presence of cytoplasmic fraction, labeling of soluble protein does proceed. Hunter et al reported that part of radioactivity accumulated in membrane fraction is transferred to soluble fraction immediately after the addition of cytoplasmic supernate (*19*) and they present a hypothesis on the mechanism of completion of protein in which active role of membrane component, phospholipid to serve more rigid orientation of activated amino acid on RNA templats is emphasized (*20*).

Incorporation of radio active amino acid into cytochrome c_{554} in *vitro* is shown and this is the great relief for the study on the mechanism of

cytochrome synthesis in bacteria. SIMPSON et al (*6, 21*) reported the net synthesis of cytochrome in calf-heart mitochondria and ^{14}C-lysine and -valine were shown to be incorporated into the molecule of cytochrome and rate of labeling of valine in C-terminals of haeme peptide much more faster than that of N-terminal one. SCHWEET et al (*22, 23*) isolated radioactive haemoglobin after the incubation of rabbit reticulocyte microscome with ^{14}C-leucine and suggested that there exists macromolecular precursor in microsome, and complete haemoglobin might be synthesized by stepwise addition of amino acid to its C-terminal. In other words the peptide chain of haemoglobin is synthesized from the N-terminal. It should be noted that SHIMURA et al (*24*) found excess labeling in the N-terminal of glycine of silk fibroin, indicating sequential synthesis from the carboxyl end of the chain. Details of experiments in *Pseudomonal-P* described in this article have been and to be published in J. Biochem. (Tokyo) (*25–30*).

These experiments have done with collaboration of following investigators.

Taxonomy. Dr. K. KOMAGATA (Institute of Applied Microbiolog, Tokyo University.)

Physiology of pyrocatechase induction. Dr. Y. MARUYAMA (ibid).

Physical and chemical analysis of macromolecular components. Dr. Y. MARUYAMA and Miss E. YOSHIDA (ibid)

Amino acid incorporation experiments *in vivo* and *in vitro*. Miss E. YOSHIDA, Dr. H. TAKAHASHI and Prof. B. MARUO (ibid).

Purification of pyrocatechase. Dr. H. SHIGEMATSU (The Sericultural experiment Station. Tokyo).

Physiological study of cytochrome formation. Dr. K. TITANI (Institute for Protein Research, Osaka University) and Dr. S. MINAKAMI (Dept. of Nutrition, Medical School, Tokyo University).

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DIFFERENTIATION OF GENUS *PSEUDOMONAS* AND RELATED AEROBIC BACTERIA

KAZUO KOMAGATA*

Institute of Applied Microbiology, University of Tokyo

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The genus *Pseudomonas* and the other related aerobic bacteria, such as *Achromobacter*, *Flavobacterium*, *Aerobacter*, *Micrococcus*, *Brevibacterium*, *Bacillus* etc., widely distribute and contribute to the cycles of elements in nature. It has been reported that genus *Pseudomonas* participate in the important role as main bacteria composed of microflora of cereals (*1*, *2*), peteroleum (*3*, *4*), natural gas (*4*, *5*), etc. *Pseudomonas* and the related bacteria are easily isolated on ordinary media from water, sewage, soil etc., as well as above mentioned sources, but detailed microbiological characteristics are not known enough to permit differentiation.

The differentiations of aerobic bacteria have been reported on the respective basis of assimilation of special compounds (*6*, *7*), sensitivity to antibiotics (*8*), and the biochemical characteristics as shown on the bacteria of *Enterobacteriaceae* (*9*, *10*, *11*). However, the bacteria differentiated by these methods are limited to special groups, and not to be adopted to *Pseudomonas* and the other related bacteria. A work by SKERMAN (*12*) is a valuable contribution, but rather complicated and not suitable if anything for the ecological studies of microflora.

For the purpose of easy differentiation of aerobic bacteria, as an aid to ecological studies of microflora, several cultural and biochemical characteristics of type cultures were tested. From the results, a key to differentiation of aerobic bacteria, mainly genus *Pseudomonas*, is reported in this paper.

EXPERIMENTAL

The genus *Pseudomonas* is nutritionally simple, and assimilates nitrate, ammonium, and amino acids as a sole source of nitrogen. Characteristic fluorescent pigments are formed by some species. From these facts, differential media are composed. Names of genera and species are mainly taken from Bergey's Manual of Determinative Bacteriology 7ed. (*13*).

Method

Bacteria are cultured in 8 ml. of liquid culture media in test-tube station-

* Present address: Central Research Laboratory, Ajinomoto Co., Inc., 2964, Suzuki-cho, Kawasaki, Kanagawa Prefecture.

arily except nutrient agar slant, and observed for growth, pigmentation etc., at 30° for 5 to 10 days.

Media

1. Nutrient agar slant

All strains tested grow well and the coloration on this medium is an important characteristic.

Table 1. Differential Media.

(1)	Nutrient agar slant		
	peptone	10 g	
	meat extract	10 g	
	NaCl	3 g	
	Agar	20 g	
		Water to make 1000 ml.	pH 7.2
(2)	Glucose-ammonium soln.		
	Glucose	10 g	
	$(NH_4)H_2PO_4$	1 g	
	KCl	0.2 g	
	$MgSO_4$	0.2 g	
		Water to make 1000 ml.	pH 7.2
		Colored with B. C. P.	
(3)	*p*-Hydroxy-benzoate soln.		
	p-hydroxy-benzoate (as Na salt)	2 g	
	NH_4NO_3	1 g	
	K_2HPO_4	1 g	
	$MgSO_4$	0.5 g	
		Water to make 1000 ml.	pH 7.2
(4)	Glucose-nitrate soln.		
	glucose	10 g	
	$NaNO_3$	1 g	
	K_2HPO_4	1 g	
	$MgSO_4$	0.5 g	
	KCl	0.2 g	
		Water to make 1000 ml.	pH 7.2
(5)	Succinate-nitrate soln.		
	Substitute 10 g of Na-succinate for glucose of glucose-nitrate soln.		
(6)	Glutamate soln.		
	glucose	10 g	
	Na-glutamate	5 g	
	K_2HPO_4	1 g	
	$MgSO_4$	0.2 g	
	KCl	0.1 g	
		Water to make 1000 ml.	pH 7.2
(7)	B. C. P. milk		
	Colored with B. C. P.		

2. Glucose-ammonium soln.

This medium is the simplest, composed of glucose and ammonium salt, with B. C. P. added as indicator. The growth and acid production from glucose are observed.

3. *p*-Hydroxy-benzoate soln.

This substance is well assimilated oxidatively by some species belonging

Table 2. Number of Bacteria Tested.

	No. of Species	No. of Strain
Pseudomonadaceae		
Pseudomonas	29	191
Xanthomonas	5	8
Protaminobacter	2	2
Aeromonas	2	2
Kluyvera	2	5
Spirillaceae		
Vibrio	2	2
Rhizobiaceae		
Agrobacterium	2	5
Achromobacteraceae		
Alcaligenes	2	4
Achromobacter	5	6
Flavobacterium	11	13
Enterobacteriaceae		
Escherichia	1	15
Aerobacter	2	12
Erwinia	2	2
Serratia	2	4
Proteus	1	1
Micrococcaceae		
Micrococcus	12	20
Sarcina	5	9
Staphylococcus	1	1
Brevibacteriaceae		
Brevibacterium	6	12
Arthrobacter	2	2
Corynebacterium		
Corynebacterium	5	13
Microbacterium	2	2
Bacillaceae		
Bacillus	10	19
	113	350

to *Pseudomonas*. The pathway of assimilation is shown by inductive enzymes (*14*). The growth in this media indicates the adaptivity and oxidability (*15*).

4. Glucose-nitrate soln.

The assimilation of nitrate is detected.

5. Succinate-nitrate soln.

Several strains prefer succinate to glucose. Occasionally gas formation due to denitrification is observed.

6. Glutamate soln.

The growth in this medium composed of glucose and glutamate shows the nutritional requirements in a broad sense. This medium is also suitable for the formation of fluorescent pigments of *Pseudomonas*.

7. B. C. P. milk

The formation of gas and acid from lactose, coagulation and peptonization of casein are the eminent characteristics of the bacteria belonging to *Enterobacteriaceae*, and others.

These media used in this paper are described in Table 1.

Microorganisms

The bacteria, belonging to 23 genera, 113 species and 350 strains, are mainly taken from type cultures kept at the Institute of Applied Microbiology, University of Tokyo, and the others are newly isolated and determined by the author. The number of bacteria tested is shown on Table 2.

RESULTS

The following characteristics were recognized on each genera.

1. Pseudomonas

Pseudomonas is Gram negative rod possessing polar flagella, and easily isolated from soil, water, cereals, etc. This genus is divided into three groups by the author (*16*); fluorescent group which forms fluorescent soluble pigments; achromogenic group which does not form any pigment; chromogenic group which forms insoluble yellow pigments. These three groups are also separated according to the characteristics evidenced in the metabolic map. Almost all strains belonged to fluorescent group grew well on the seven media mentioned, utilized characteristically *p*-hydroxy-benzoate, and formed fluorescent pigments in glutamate soln. Reaction to B. C. P. milk was divided into three types, that is, alkaline-peptonization type, alkaline type, and acid type. Achromogenic group was biochemically less active than fluorescent group; several strains did not grow in glutamate soln. *Ps. stutzeri* and *Ps. rathonis* grew well in succinate-nitrate soln. and showed gas formation due to denitrification. Reaction to milk was almostly alkaline type except alkaline-peptonization type of *Ps. putrefaciens* and *Ps. alcaligenes*. Chromogenic group included various types of reaction to milk. *Ps. perlurida* and *Ps. trifolii* coagulated, and *Ps. lacunogenes* and *Ps. cerealis* peptonized.

2. Xanthomonas

Xanthomonas is Gram negative rod which forms yellow colonies, and similar to chromogenic group of *Pseudomonas*. However, the former was less active than the latter.

3. Vibrio

The non-pathogenic *Vibrio* is usually isolated from water. Two strains testes were biochemically inactive.

4. Protaminobacter

The strains tested were biochemically inactive.

5. Aeromonas

The biochemical characteristics were similar to *Aerobacter* without flagellation. Slowly liquefied milk.

6. Kluyvera (*17*)

Kluyvera was biochemically similar to *Escherichia*.

7. Alcaligenes

Alcaligenes was very similar to *Achromobacter*. *Al. faecalis* grew in glucose-ammonium but reaction turned to alkaline.

8. Achromobacter

Achromobacter was biochemically similar to achromogenic group of *Pseudomonas*, grew poorly in synthetic media. Generally reaction to milk were alkaline types, rarely acid types but not coagulated.

9. Flavobacterium

A yellow coloration was shown on nutrient agar slants, but biochemical characteristics were similar to *Achromobacter*. This genus biochemically corresponed to chromogenic group of *Pseudomonas*.

10. Agrobacterium

Agrobacterium is known as plant parasite and powerful oxidizer, and widely distributes in soil. The flagellation is reported as intermediate type (*13*). The strains tested grew well in synthetic media, and assimilated *p*-hydroxy-benzoate characteristically.

11. Escherichia

Escherichia formed gas and rapidly coagulated milk. In glucose-ammoium soln., growth was hardly recognized, but acidification of medium occured rapidly, and the medium completely turned to yellow. This is different from the acidification by *Pseudomonas* which slowly turns to yellow from top to bottom.

12. Aerobacter

Aerobacter was similar to *Escherichia*, but more active than the latter. It grew in glutamate soln. and succinate-nitrate soln. Some strains assimilated *p*-hydroxy-benzoate.

13. Serratia

Red coloration on nutrient agar slant was characteristic. Slowly coagu-

lated milk, and did not grow in synthetic media.

14. Erwinia

Erwinia is known as plant-parasite, divided into two groups by coloration. The strains tested were biochemically similar to *Aerobacter*.

15. Proteus

A strain tested was biochemically inactive.

16. Micrococcus

Micrococcus was biochemically inactive than the bacteria of Gram negative rod. The coloration on nutrient agar slant was characteristic of each species. Slowly acidified milk but not coagulated compactly.

17. Sarcina

Sarcina was biochemically similar to *Micrococcus* except cell forms.

18. Staphylococcus

A strain tested was biochemically inactive.

19. Brevibacterium

Breuibacterium showed the various coloration on nutrient agar slant, and nutritionally complex.

20. Arthrobacter

The strains tested were comparatively active for assimilation.

21. Corynebacterium

The nutritional requirements of pathogenic strains were rather complex, but soil forms were simple.

22. Microbacterium

Microbacterium was biochemically similar to *Brevibacterium* or *Corynebacterium*.

23. Bacillus

B. megaterium, *B. subtilis*, and *B. pumilus* grew in glutamate soln. However, the other bacilli only showed scanty growth on nutrient agar slants. No strain assimilated *p*-hydroxy-benzoate.

Experimental results are shown on Table 3.

From the results a key to aerobic bacteria was described on Table 4. The fluorescent pigments forming bacteria is limited to fluorescent group of *Pseudomonas*, so this group is easily differentiated from the others. Another group corrsponding to soluble pigment non-forming bacteria, is sub-divided according to reaction to milk. The bacteria, which coagulated milk and rapidly formed acid fermentatively, are *Escherichia*, *Aerobacter*, *Erwinia* etc., of *Enterobacteriaceae*, and *Kluyvera*, *Ps. perlurida* and *Ps. trifolii* of *Pseudomonadaceae*. In the group which slowly acidified milk but not coagulated, *Ps. stutzeri*, *Ach. superficialis* and several strains of *Micrococcus* and *Brevibacterium* are included.

A group of milk alkaline type is divided into two by assimilation of *p*-hydroxy-benzoate, and furthermore differentiated both by growth in glutamate soln. and by coloration on nutrient agar slants.

Table 3-1. Assimilation of Aerobic Bacteria.

	Bacterial No.	glucose-ammonium		*p*-hydroxy-benzoate	glucose-nitrate	succinate-nitrate	glutamate	B. C. P. milk
		G	A					
Pseudomonadaceae								
Pseudomonas								
fluorescent group								
Ps. aeruginosa	(17)	+	+	+	+	+	+p	Al. P.
Ps. azotoformans	(20)	+	+	+	+	+	+p	Al. P.
Ps. schuylkilliensis	(22)	+	+	+	+	+	+p	Al. P.
Ps. chlororaphis	(2)	+	+	+	+	+	+p	Al. P.
Ps. aureofaciens	(1)	+	+	+	+	+	+p	Al. P.
Ps. pavonacea	(7)	+	+	+	v	v	+p	Al. P.
Ps. ovalis	(27)	+	+	+	v	v	+p	Al.
Ps. nitroreducens	(6)	+	+	+	+	+	+p	Al.
Ps. rugosa	(3)	+	+	+	+	+	+p	Ac.
Ps. fulva	(6)	+	+	+	−	−	+p	Al.
Ps. cinnamona	(3)	+	+	+	+	+	+p	Al. P.
Ps. straminea	(1)	+	+	+	−	−	+p	Al.
achromogenic group								
Ps. putrefaciens	(4)	−	−	−	−	+	+	Al. P.
Ps. alcaligenes	(5)	−	−	−	−	−	−	Al. P.
Ps. stutzeri	(6)	+	+	−	v	+	+	Un. or sl. Ac.
Ps. rathonis	(6)	−	−	−	v	+	+	Al.
Ps. denitrificans	(4)	−	+	−	−	+	−	Al.
Ps. dacunhae	(1)	−	−	+	−	+	+	Al.
Ps. desmolytica	(12)	−	−	+	−	+	+	Al.
Ps. iners	(3)	−	−	−	−	−	−	Un. or sl. Al.
Ps. riboflavina	(1)	−	−	−	−	−	−	Un. or sl. Al.
Ps. diminuta	(1)	−	−	−	−	−	−	Un. or sl. Al.
chromogenic group								
Ps. perlurida	(7)	+	+	−	+	+	+	Ac. Coag.
Ps. trifolii	(4)	+	+	−	−	+	+	Ac. Coag.
Ps. cerealis	(4)	+	+	−	−	−	+	Al. P.
Ps. melanogenum	(4)	−	−	−	−	−	−	Al. P.
Ps. lacunogenes	(12)	+	+	+	+	+	+	Al. P.
Ps. ochracea	(2)	−	−	+	−	−	+	Al.

Table 3-2.

	Bacterial No.	glucose-ammonium		*p*-hydroxy-benzoate	glucose-nitrate	succinate-nitrate	glutamate	B. C. P. milk
		G	A					
Pseudomonadaceae								
Xanthomonas								
X. campestris	(1)	−	−	−	−	−	−	Un.
X. begoniae	(1)	−	−	−	−	+	+	Al. P.
X. pruni	(1)	−	−	−	−	+	±	Al. P.
X. oryzae	(4)	−	−	−	−	−	−	Un.
X. malvacearum	(1)	−	−	−	−	+	−	Un.
Protaminobacter								
P. alboflavum	(1)	+	−	−	+	+	+	Al.
P. rubrum	(1)	−	−	−	+	+	+	Al.
Aeromonas								
A. hydrophila	(1)	+	+	−	+	+	+	Ac. P.
A. punctata	(1)	+	+	−	±	+	+	Ac. P.
Kluyvera								
K. citrophila	(3)	±	+	−	−	−	−	Ac.
K. non-citrophila	(2)	±	+	−	−	−	−	Ac.
Spirillaceae								
Vibrio								
V. metchinikovii	(1)	−	−	−	−	−	−	Un.
V. tyrogenes	(1)	−	−	−	−	−	−	Un.
Rhizobiaceae								
Agrobacterium								
A. tumefaciens	(3)	+	+	+	+	+	+	Al.
A. radiobacter	(2)	+	+	+	+	+	+	Al. P.
Achromobacteraceae								
Alcaligenes								
A. faecalis	(3)	+	−	−	v	+	+	Al.
A. viscolactis	(1)	−	−	−	−	+	+	Ac.
Achromobacter								
A. liquidum	(1)	−	−	−	−	−	−	Al.
A. cycloclastes	(1)	+	+	−	+	+	+	Al.
A. pestifer	(1)	−	−	−	−	−	−	Al.
A. eurydice	(1)	−	−	−	−	+	−	Un.
A. superficialis	(2)	v	+	−	−	v	v	Ac.

Table 3-3.

	Bacterial No.	glucose-ammonium G	glucose-ammonium A	*p*-hydroxy-benzoate	glucose-nitrate	succinate-nitrate	glutamate	B. C. P. milk
Achromobacteraceae								
Flavobacterium								
F. aquatile	(2)	−	−					Al. P.
F. sewanense	(1)	−			−		−	Un.
F. lactis	(1)	−		−	−		−	Un.
F. arborescens	(1)	−	−	−	−	−	−	Al. P.
F. suaveolens	(1)	−	−	−	−	−	−	Al.
F. citreum	(1)	−	−	−		−	−	Un.
F. fuscum	(1)	−	−	−	−	−	−	Al. P.
F. rigense	(1)	−	−	−	−	−	−	Al.
F. sulfureum	(1)	−		−	−	−	−	Al. P.
F. ferrugineum	(1)	+		−			+	Al.
F. dormitator	(2)	+	+			−	+	Al.
Enterobacteriaceae								
Escherichia								
E. coli	(15)	−	+	−	−	∓	∓	Ac. Coag.
Aerobacter								
A. aerogenes	(6)	+		v	−	+	+	Ac. Coag.
A. cloacae	(6)	+	+	−	−	+	+	Ac. Coag.
Erwinia								
E. carotovora	(1)	−	+	−		+	+	Ac. Coag.
E. aroideae	(1)	−	+	−	−	+	+	Ac. Coag.
Serratia								
S. marcescens	(3)	−	+	−	−	−	+	Ac. Coag.
S. plymuthica	(1)	−	+	−	−	−	−	Ac. Coag.
Proteus								
P. vulgaris	(1)	−	−	−	−		−	Un.

Table 3-4.

	Bacterial No.	glucose-ammonium		*p*-hydroxy-benzoate	glucose-nitrate	succinate-nitrate	glutamate	B. C. P. milk
		G	A					
Micrococcaceae								
Micrococcus								
M. luteus	(3)	−	−	−	−	−	−	Un. or sl. Al.
M. freudenreichii	(1)	−	−	−	−	−	−	Ac.
M. varians	(3)	−	v	−	−	−	−	Al.
M. roseus	(1)	−	−	−	−	−	−	Un.
M. rubens	(1)	−	−	−	−	−	−	Un.
M. candidus	(2)	−	−	−	−	−	−	Ac.
M. conglomeratus	(4)	−	−	−	−	−	−	Un.
M. citreus	(1)	−	−	−	−	−	−	Ac.
M. epidermides	(1)	−	−	−	−	−	−	Al.
M. flava	(1)	−	−	−	−	−	−	Al.
M. caseolyticus	(1)	−	−	−	−	−	−	Un. or sl. Al.
M. lysodeikticus	(1)	−	−	−	−	−	−	Un.
Sarcina								
S. lutea	(1)	−	−	−	−	−	−	Un.
S. albida	(1)	−	−	−	−	−	−	Un.
S. aurantiaca	(1)	−	−	−	−	−	−	Un.
S. variabilis	(1)	−	−	−	−	−	−	Un.
S. flava	(5)	−	−	−	−	−	v	Un.
Staphylococcus								
S. aureus	(1)	−	−	−	−	−	−	Un.
Brevibacteriaceae								
Brevibacterium								
B. ammoniagenes	(2)	−	−	−	−	−	−	Al.
B. helvolum	(4)	−	−	−	−	−	−	Un.
B. acetylicum	(2)	−	−	−	−	−	−	Al.
B. sulfureum	(1)	−	−	−	−	−	−	Un.
B. lipolyticum	(2)	−	−	−	+	+	+	Al. P.
B. imperiale	(1)	−	+	−	−	−	−	Ac.
Arthrobacter								
A. simplex	(1)	+	+	−	+	+	+	Al. P.
A. ureafaciens	(1)	+	−	+	+	+	+	Al.

Table 3-5.

	Bacterial No.	glucose-ammonium G	glucose-ammonium A	*p*-hydroxy-benzoate	glucose-nitrate	succinate-nitrate	glutamate	B. C. P. milk
Corynebacteriaceae								
Corynebacterium								
C. hoagi	(1)	−	−	−	−	−	−	Un.
C. facians	(1)	+	−	+	+	+	+	Al.
C. equi	(1)	−	−	−	−	−	−	Un.
C. hydrocarboclastus	(5)	+	+	−	−	+	+	Al.
C. oleophilus	(5)	−	v	−	−	+	−	Al.
Microbacterium								
M. flavum	(1)	−	+	−	−	−	−	Un.
M. lacticum	(1)	−	+	−	−	−	−	Ac.
Bacillaceae								
Bacillus								
B. subtilis	(4)	+	+	−	v	+	+	Al. P.
B. coagulans	(2)	−	−	−	−	−	−	Un. or sl. Al.
B. cereus	(3)	−	−	−	−	−	−	Al. P.
B. cereus var. mycoides	(1)	−	−	−	−	−	−	Al. P.
B. firmus	(1)	−	−	−	−	−	+	Un.
B. circulans	(2)	−	−	−	−	−	−	Un. or sl. Al.
B. sphericus	(1)	−	−	−	−	−	−	Un. or sl. Al.
B. pumilus	(1)	+	+	−	−	−	+	Al. P.
B. megaterium	(3)	+	+	−	−	−	+	Al. P.
B. brevis	(1)	−	−	−	−	−	+	sl. P.

Abbreviation:

+	positive growth
−	negative growth
v	variable growth by each strains
p	formation of soluble pigments
Al.	alkaline
P.	peptonization
Ac.	acid
Un.	unchange
Coag.	coagulation
sl.	slightly
G.	growth
A.	acid formation

Table 4. Differential Key of Aerobic Bacteria

(I)	Soluble pigment formed (yellow, green, orange, fluorescent).		
	p-Hydroxy-benzoate attack.		
	greenish yellow pigment produced		
	milk alkaline		
	succinate-nitrate assimilate		
		Ps. ovalis	(23)*
		Ps. nitroreducens	(6)
	succinate-nitrate not assimilate		
	brown on slant	Ps. ovalis	(4)
	yellow on slant	Ps. fulva	(6)
		Ps. straminea	(1)
	milk peptonize		
	succinate-nitrate assimilated		
		Ps. aeruginosa	(17)
		Ps. azotoformans	(20)
		Ps. schuylkilliensis	(22)
		Ps. chlororophis	(2)
	nutrient slant becoming brownish		
		Ps. pavonacea	(7)
	milk acid		
		Ps. rugosa	(3)
	orange pigment produced		
	milk peptonize		
	brown on slant	Ps. aureofaciens	(1)
	yellow on slant	Ps. cinnamona	(3)
(II)	Soluble pigment not formed		
	milk acid		
	readily milk coagulate		
	glutamate assimilate		
	p-hydroxy-benzoate attack		
		Aerobact. aerogenes	(2)
	p-hydroxy-benzoate not attack		
	white to pale brown on slant		
		Aerobact. aerogenes	(4)
		Aerobact. cloacae	(4)
		Aeromonas hydrophila	(1)
		Aeromonas punctata	(1)
		Erwinia aroideae	(1)
		Erw. carotovora	(1)
	yellow on slant	Ps. perlueida	(7)
		Ps. trifolii	(4)
	pink to red on slant		
		Serratia marcescens	(4)
		S. plymuthicum	(1)
	glutamate not assimilate		
	readily acid but not grow or scanty growth in glucose-ammonia.		
	p-hydroxy-benzoate not attack.		

Escherichia coli (15)
Kluyvera citrophila (3)
K. non-citrophila (2)

very slowly milk coagulate or not
glutamate assimilate
succinate-nitrate assimilate
Ps. stutzeri (6)
Ach. superficialis (1)
succinate-nitrate not assimilate
Micrococcus candidus (2)
M. caseolyticus (1)
M. freudenreichii (1)
M. citreus (1)
Al. viscolactis (1)
Microbact. lacticum (1)
Brevi. imperiale (1)
Ach. superficialis (1)

milk alkaline
p-hydroxy-benzoate attack
glutamate assimilate
succinate-nitrate assimilate
Agro. tumefaciens (3)
Cory. fascians (1)
Arth. ureafaciens (1)
glucose-ammonia not assimilate
brown on slant Ps. dacunhae (1)
Ps. desmolytica (12)
succinate-nitrate not assimilate
yellow on slant Ps. ochracea (2)
p-hydroxy-benzoate not assimilate
glutamate assimilate
succinate-nitrate assimilate
white to pale brown on slant
Ps. rathonis (6)
Ach. cycloclastes (1)
Al. faecalis (3)
pale pink to red on slant
Prot. ruber (1)
Cory. hydrocarboclastus (5)
succinate-nitrate not assimilate
brown on slant Ach. pestifer (1)
yellow no slant Flavo. ferrugineum (1)
Flavo. dormitator (2)
M. flava (1)
glutamate not assimilate
succinate assimilate
Ps. denitrificans (4)
Cory. oleophilus (5)
succinate not assimilate

brown on slant
- Ps. iners (3)
- Ps. riboflavina (1)
- Ps. diminuta (1)
- Ach. liquidum (1)
- Brevi. ammoniagenes (1)

yellow on slant
- Flavo. suaveolens (1)
- Flavo. rigenes (1)
- M. varians (3)
- M. luteus (3)
- Brevi. ammoniagenes (1)

transparent or scanty growth on slant
- Bac. circulans (2)
- Bac. coagulans (2)
- Bac. sphericus (1)
- M. epidermides (1)

milk peptonize

p-hydroxy-benzoate attack

glutamate assimilate

succinrte-nitrate assimilate

brown on slant
- Agro. radiobacter (2)

yellow on slant
- Ps. lacunogenes (12)

p-hydroxy-benzoate not attack

glutamate assimilate

white or pale brown on slant
- Bac. megaterium (3)
- Bac. subtilis (4)
- Bac. pumilus (1)
- Bac. brevis (1)
- Brevi. lipolyticum (2)
- Arth. simplex (1)

yellow on slant
- X. begoniae (1)
- X. pruni (1)

glutamate not assimilate

brown on slant
- Ps. alcaligenes (5)
- Ps. putrefaciens (4)
- Bac. cereus (3)
- Bac. cereus var. mycoides (1)

yellow on slant
- Ps. cerealis (4)
- Ps. melanogenum (4)
- Flavo. aquatile (2)
- Flavo. arborescens (1)
- Flavo. fuscum (1)
- Flavo. sulfureum (1)

milk unchange, reaction almostly neutral.

glutamate assimilate

succinate-nitrate assimilate

brown on slant
- Ach. eurydice (1)

yellow on slant
- Sarcina flava (2)

glutamate not assimilate		
white to pale brown on slant		
	V. tyrogenes	(1)
	V. metchinikovii	(1)
	Pr. vulgaris	(1)
	Sarcina albida	(1)
	Brevi. helvolum	(4)
	Cory. hoagi	(1)
	Bac. firmus	(1)
yellow on slant	X. campestris	(1)
	X. malvacearum	(1)
	X. oryzae	(1)
	Flavo. lactis	(1)
	Flavo. sewanense	(1)
	Flavo. citreum	(1)
	M. lysodeikticus	(1)
	M. conglomeratus	(5)
	Sarcina lutea	(1)
	Sarcina variabilis	(1)
	Sarcina flava	(3)
	Brevi. sulfureum	(1)
	Microbact. flavum	(1)
orange on slant	M. roseus	(1)
	M. rubescens	(1)
	Staph. aureus	(1)
	Sarcina aurantiaca	(1)
	Brevi. acetylicum	(2)
	Cory. equi	(1)

Abbreviation of generic name:

Ps.	Pseudomonas
X.	Xanthomonas
Prot.	Protaminobacter
K.	Kluyvera
V.	Vibrio
Agro.	Agrobacterium
Ach.	Achromobacter
Al.	Alcaligenes
Flavo.	Flavobacterium
Aerobact.	Aerobacter
S.	Serratia
Pr.	Proteus
M.	Micrococcus
Staph.	Staphylococcus
Brevi.	Brevibacterium
Arth.	Arthrobacter
Cory.	Corynebacterium
Microbact.	Microbacterium
Bac.	Bacillus

* No. of strain tested

The bacteria which liquified milk are also divided into two by assimilation of *p*-hydroxy-benzoate. Those which assimilate glutamate and not *p*-hydroxy-benzoate are chiefly belonged to Gram positive rods. The chromogenic group of *Pseudomonas* and *Flavobacterium* of this group are settled on same position by nutritional requirements.

The bacteria belonging to a group of milk-unchanging are biochemically less active. Almost all *Micrococcus*, *Sarcina*, *Brevibacterium* etc. belonged to this group.

DISCUSSION

The aerobic bacteria, mainly genus *Pseudomonas*, are easily differentiated by this procedure. The bacteria of Gram positive which showed nutritionally complex requirements are not readily differentiated. For further differentiation of this group, other characteristics on the basis of cell morphology, ecology, and biochemistry should be studied. As an aid to the ecological research of microorganisms, it would be more satisfactory that colonies on several media not only counted but also many isolates are differentiated by this procedure. In other words, microflora should be discussed on the basis of bacterial genera or species which indicate quality, with the numbers of colonies which show the quantity. For, the bacteria showing the same nutritional requirements are treated equally, regardless of genera of species composed of microflora.

From the view-points of biochemical and nutritional characteristics of aerobic bacteria, some relationships are found between the genera. In respects to genus *Pseudomonas*, achromogenic group is similar to *Achromobacter* and *Alcaligenes*, chromogenic group is similar to *Flavobacterium*. *Agrobacterium* is oxidative and very similar to fluorescent group of *Pseudomonas* except the non-formation of soluble fluorescent pigment and peritrichous flagellation. However, on the basis of its oxidability, *Agrobacterium* is belonged to *Pseudomonadaceae* by Prevot (*18*). By the same manner, biochemically *Kluyvera* and *Aeromonas* shoud be belonged to *Enterobacteriaceae*, as these are only differentiated by flagellation. On the other hand, *Erwinia* is parasitic to plant, although it belonged to *Enterobacteriaceae* that are mainly found in animals. From the ecological view-points, yellow-colonies forming species of this genus are transferred to *Flavobacterium* by Brisou (*19*). As above mentioned, some genera of different family of Bergey's system are biochemically similar each other, and the other genera of same family are nutritionally far apart. On these genera, a confusion of bacteria taxonomy would seemingly result.

The motility and flagellar arrangement are important characteristics for bacterial taxonomy. From the results obtained, a relationship between flagellation and biochemical similarity of aerobic Gram negative bacteria is summarized on Table 5. On the basis of flagellation and biochemical similarity, bacteria are grouped from left to right, and vertically arranged

Table 5. Relationship between flagellar arrangement and bacterial genera.

Polar flagellation	Peritrichous flagellation	Non-motile
Pseudomonas (fluorescent group)*	Agrobacterium	
Pseudomonas (achromogenic group)*	Achromobacter Alcaligenes	Acinetobacter
Pseudomonas (chromogenic group)*	Flavobacterium	Empediobacter
Phytobacterium**		Aplanobacter
Kluyvera***	Escherichia	Escherichia
Aeromonas	Cloacae****	Aerobacter

* K. KOMAGATA (*16*) *** T. ASAI (*17*)
** J. BRISOU (*19*) **** F. KAUFFMAN (*25*)

by Bergey's system and newly reported genera and groups.

As shown on table, it is biochemically more resonable that some genera should be grouped by horizontal lines because they are very alike except flagellation.

The flagellar variation of polar to peritrichous (*20*, *21*), or mixed flagellation (*20*, *22*), or transduction of flagella (*23*), and the facts that flagellation is altered by the composition of media or enviromental factors (*24*) are reported on several genera. Comparing with the biochemical activities, morphogenesis of bacteria belonging to *Eubacteriale* is not so developed. On the taxonomical studies of these Gram negative bacteria, it is necessary to base not only on morphological characteristics, but also on biochemical characteristics, such as type of energy metabolism, oxidative or fermentative, assimilationof nitrogen etc. These results point out that taxonomical value of flagellar arrangement of these bacteria are less than that of other morphological characteristics.

On the studies of microflora of cereals, peteroleum, natural gas etc., satisfactory results are obtained by this procedure (*2*, *3*, *4*, *5*).

SUMMARY

As an aid to ecological research of microorganisms, a differentiation of aerobic bacteria, mainly genus *Pseudomonas*, was studied. On 28 genera, 114 species and 350 strains of type cultures, cultural and biochemical characteristics, such as assimilation of nitrate, ammonium, glutamate, succinate, glucose, and *p*-hydroxy-benzoate, reaction to B. C. P. milk, formation of soluble pigments, coloration on nutrient agar slant etc., were tested. The results are shown on Table 3, and the author described a differential key of aerobic bacteria, which illustrated on Table 4. The bacteria belonged to Gram negative group, such as *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Escherichia*, *Aerobacter* etc., are biochemically simple, and would be differ-

entiated by this key. On the other hand, the bacteria of Gram positive, such as *Micrococcus*, *Sarcina*, *Brevibacterium*, *Bacillus* etc., are nutritionally more complex than that of Gram negative group, and would not easily differentiated by this procedure. Furthermore, some problems of bacterial taxonomy were discussed on the basis of the relationship between bacterial flagellation and biochemical characteristics.

ACKNOWLEDGEMENTS

The author is very grateful to Prof. Dr. IIZUKA of this institute for his kind guidance throughout the course of this study. He is also grateful to the member of The Division of Taxonomy and Type Culture Collection of this institute for their technical assistance.

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Abstract

Reports of studies conducted by members of the Institute of Applied Microbiology, University of Tokyo, published elsewhere are abstracted

Nature, **186**, 567-568 (1960)

Isolation of Mutants of *Bacillus subtilis* requiring D-Glutamic Acid

By Haruo MOMOSE and Yonosuke IKEDA

Division of Microbial Genetics

Four types of mutants requiring D-glutamic acid. were induced by X-ray irradiation from a wild strain of *Bacillus subtilis K*. The mutants differed in terms of the growth responses on six amino acids; D- and L-glutamate, aspartate, and alanine.

Nature **186**, 818 only (1960)

Physiology of D-Glutamic Acid-requiring Mutants of *Bacillus subtilis*

By Haruo MOMOSE and Yonosuke IKEDA

Division of Microbial Genetics

A mutant D-glutamate, D-aspartate or D-alanine, exhibited when incubated in absence of the amino acid the so-called unbalanced growth death after an appreciable residual growth, meanwhile other mutants requiring D- or L-glutamate or aspartate survived under similar conditions. The experimental results imply the importance of D-glutamate as a constituent of cell wall.

Biochem. Biophys. Acta **41**, 180-181 (1960)

Stimulation of genetic transformation in *Bacillus subtilis* by biologically inactive DNA and polyphosphate

By Masamichi KOHIYAMA and Hiuga SAITO

Division of Microbial Genetics

The velocity of linear increase with time of transformants is a function of the competence of the recipient cells. The competence was improved by the treatment of cells with biologically inactive DNA or polyphosphate, prior to the addition of transforming DNA. These results were interpreted in terms of the induction of the ability of uptaking transforming DNA.

Nature **187**, 168-169 (1960)

Appearance of New Deficient Characters in Streptomycin-resistant Mutants of *Bacillus subtilis*

By Masamichi KOHIYAMA and Yonosuke IKEDA

Division of Microbial Genetics

When the cells of *B. subtilis K* were plated onto a medium containg a high concentration of streptomycin, a portion of the resistant mutants appeared on the plate were found to have acquired new auxotrophic characters. The new mutant is dependent on histidine, thiamine or uracil.

C. R. Soc. Biol. **165**, 690 (1960)

Recherches génétiques sur le *Bacillus subtilis*

By Masamichi KOHIYAMA

Division of Microbial Genetics

This paper comprises a brief review on the genetic interaction and the mutation in nutrient dependent and streptomycin resistant clones of *Bacillus subtilis K*.

Antarctic Record No. 11
Special Publication of the Antarctic Symposium in Tokyo, (1960) 161

On the Microflora of the Antarctic Zone. Part I. On the Bacterial Flora

By Hiroshi IIZUKA
Division of Taxonomy

Bacteria and fungi were isolated from the materials collected aseptically and brought back in the refrigerators (1-3°C).

With regard to bacteria, 846 strains were isolated and identified. Of those, *Brevibacterium* were 386, *Pseudomonas* and *Achromobacter* 383 and others 77. As for low temperature bacteria, *Brevibacterium*, which should be called obligate psychrophiles and whose most favorable temperature for growth is somewhere between 5°C and 10°C, were obtained. And many *Pseudomonas* were obtained, which can live also at low temperature, since the scope of temperature favorable for living are of a wide range. Bacteria which have strict requirements in nutrition though of low temperature quality were found in pieces of the seal flesh.

Nippon Nôgei-Kagaku Kaishi,
Vol. 34, No. 6 (1960) 469-472

Oxidation of Progesterone and Reichstein's Compound S by Genus *Pseudomonas* and Others. Part II. Studies on Steroid and Microorganisms

By Hiroshi IIZUKA and Atsushi NAITO
Division of Taxonomy

It was considered that there were some possibilities in using bacteria, among various microorganisms, for microbiological oxidation of steroids. Optimal conditions of culture were examined prior to oxidation of steroids. The most important point in this reaction was that the addition of a substrate (methanol solution) and inoculation of bacteria to the medium be made at the same time.

Oxidation ability was tested on progesterone and Reichstein's compound S with 162 strains of *Pseudomonas* sp. and 24 strains of *Micrococcus* sp. preserved in this laboratory.

Previously, a proposal was made to classify the genus *Pseudomonas* into three large groups, the fluorescent-type, achromonogenic-type, and chromogenic-type *Pseudomonas*. Achromogenic-type *Pseudomonas* had stronger ability to oxidize steroids than other types. Therefore, this classification of *Pseudomonas* genus seemed to be appropriate even from the characteristics of various strains on oxidation ability of steroids.

Nippon Nôgei-Kakaku Kaishi,
Vol. 34, No. 6 (1960) 472-475

Production of 6β, 14α-Dihydroxyprogesterone and 6β-Hydroxycomp. S by Bacteria. (Studies on Steroid and Microorganisms. Part III).

By Hiroshi IIZUKA, Atsushi NAITO,
Eiji OHKI, Yoshihiro SATO
and Masako HATTORI
Division of Taxonomy

Progesterone and Reichstein's comp. S were compared for oxygenation ability employing various bacteria preserved in our laboratory.

As the results of this experiment, one of these strains (*Achromobacter* sp. strain K 40-5) had stronger oxygenation ability not only on progesterone but also on comp. S.

After examining culture conditions, mass cultures were performed under the best condition.

The oxidation products were 6β, 14α-dihydroxyprogesterone [m.p.=245° $[\alpha]_D$+125 (c=1.0, $CHCl_3$)], and 6β-Hydroxycomp. S [m.p.=224-226°, $[\alpha]_D$+60.5° (c=0.63, EtOH),] which were gained by bacteria for the first time.

Journal of Japanese Society of Food and Nutrition, **13**, 114-116 (1960)

Studies on the Royal Jelly (Part I) On the Vitamin Contents in Royal Jelly

By Yohnosuke Koyama and Hiroshi Iizuka

Division of Taxonomy

The following 10 vitamins, thiamine, riboflavin, vitamin B_6 group, niacin, pantothenic acid, folic acid, biotin, vitamin B_{12}, inositol, and acetylcholin contained in domestic royal jelly (Aomori) were determined by the microbioassay method.

Journal of Japanese Society of Food and Nutrition, **14**, 5-7 (1961)

Studies on the Royal Jelly (Part II) On the Amino acid and Vitamin Contents in Royal Jelly

By Hiroshi Iizuka and Yohnosuke Koyama

Division of Taxonomy

The following ten amino acids, arginine, cystine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane and valine and ten vitamins, thiamine, riboflavin, vitamin B_6 group, niacin, pantothenic acid, folic acid, biotin, vitamine B_{12}, inositol and acetylcholin contained in fresh and four months storage royal jelly were determined by the microbioassay method.

Hakko Kogaku Zasshi, **39**, 1 (1961)

Microbiological Studies on Miso (I). On the Change in Microflora in the Process of Miso-brewing

By Wataru Susukida, Fusanosuke Masuko and Toranozyo Matsuo

Division of Physiology

The authors studied the changes of microflora in the process of miso-brewing by the dilution plate method using special selective media. The chemical changes and the vicissitude of enzyme activities in the above process were also investigated. The results were as follows.

1) Anaerobic bacteria, mostly lactic acid bacteria, and yeasts decreased gradually in the process of miso-brewing.

2) Total count of aerobic bacteria decreased in the early stage of brewing until 150 days, and then became stationary. In the later stage, most of bacteria were found to be Bacilli.

3) As the amount of total acid increased, pH decreased. However, increase in number of lactic acid bacteria was not observed.

4) Enzyme activities decreased gradually at the begining of brewing, and increased in summer season (i.e. in the middle phase of brewing), although the change in microflora did not correlate with the increase in enzyme activities.

5) Highly halotorelant tetracoccus (lactic acid former) was isolated from miso. It was identified as *Pediococcus halophilus* and could be distinguished from *P. sovae* found in soy-mash based on the difference in fermentability of sucrose and dextrin.

Hakko Kogaku Zasshi, **39**, 8, (1961)

Microbiological Studies on Miso (II). On the Rôle and the Existing form of *Bacillus subtilis* in Miso

By Wataru Susukida, Fusanosuke Masuko and Toranozyo Matsuo

Division of Physiology

The rôle of Bacillus which was ascertained as the major group of bacteria found in miso was investigated, and the following results were obtained.

1) Most of *Bacillus* cells in miso was found to exist in vegetative form. This finding makes a striking contrast to the case of soy-mash where bacillus cells are found in spore form.

2) Protease and amylase activities in koji and miso were compared with those produced by *B. subtilis* isolated frrm miso. It was inferred from the results that *B. subtilis* plays an important rôle in maturing process.

3) All *Bacilli* isolated from miso were identified as *B. subtilis*, but differd from *B. subtilis* strains isolated by other workers in some physiological characteristics. Strains B-1 and B-35 were incapable of utilizing citric acid and failed to grow on glucose nitrate agar, while strains B-26 and B-27 were lactose fermenters.

Hakko Kogaku Zasshi, **39**, 14 (1961)

Microbiological Studies on Miso (III). Spore-forming Ability of *Bacillus subtilis* in Miso

By Seiichi NASUNO, Wataru SUSUKIDA and Toshinobu ASAI

Division of Physiology

The spore-forming abilities of three strains of *Bacillus subtilis* isolated from miso and *B. subtilis* NRRLB-558 were studied under various pH's and NaCl concentrations. On a miso-extract agar slant no effect of pH was observed between pH 4.5 and 8.0. In liquid medium all strains had an optimum pH for spore-formation around pH 5.0-6.0, coinciding with that for growth. With increasing concentrations of NaCl the spore-forming abilities decreased. It is concluded that the majority of spore-forming bacilli in miso are in the form of vegetative cells.

Nippon Nôgei-kagaku Kaishi, **35**, 292 (1961)

Effects of Peptides on the growth of *Leuconostoc mesenteroides* p-60

By Ko AIDA, Tadayuki KAJIWARA and Toshinobu ASAI

Division of Physiology

Among various glycyl peptides examined for the glycine-replacing ability in the growth of *Leuconostoc mesenteroides* p-60, glycylglycine, DL-alanylglycine, L-leucylglycine, and glycyl-L-leucine gave better response than glycine at low levels of concentration. With resting cells peptidase activity towards these peptides was proved. The presence of an unusually large amount of serine inhibited the growth of organism at lower concentrations of glycine, but did not do so when glycine was replaced with one of those glycine peptides.

Chem. and Pharm. Bull., **8**, 1056 (1960)

Microbiological Transformation of Thebaine into 14-Hydroxycodeinon by Several Molds

By Kazuaki IIZUKA, Shigenobu OKUDA, Ko AIDA, Toshinobu Asai, Kyoshuke TSUDA, Michihiro YAMADA and Isawo SEKI

Division of Physiology

During the studies of transformation of morphine-alkaloids by various microorganisms, some molds were found to convert thebaine into 14β-hydroxycodeinone with good yield. The recrystallized hydroxylation product was identical with authentic 14β-hydroxycodeinone in m. p. (271-272°), IR- and UV-spectra, and paper chromatographic behavior. Molecular formula obtained $C_{18}H_{19}O_4N$. Under different conditions thebaine was converted not only to 14β-hydroxycodeinone, but also to 14β-hydroxycodeine.

Bull. Agr. Chem. Soc. Jap., **24**, 196 (1960)

Studies on Oxidative Fermentation. Part XIX. Catabolism of K-5-ketogluconate by Acetobacter suboxydans. II

By Haruyoshi MUROOKA, Yukashi KOBAYASHI and Toshinobu ASAI

Division of Physiology

By the cell suspension of *Acetobacter suboxydans* ATCC 621 acetic, succinic, glycollic, pyruvic, and α-ketoglutaric acids are formed from 5-ketogluconate under aerobic conditions in the presence of a trace amount of glucose. α-Ketoglutaric acid did not undergo further oxidation and accumulated up to 3% of 5-ketogluconate supplied. The dried cell capable of oxidizing 5-

ketogluconate did not oxidize the members of the TCA cycle except oxalacetic acid, suggesting non-participation of the TCA cycle in the degradation process of 5-ketogluconate. Glycolaldehyde was oxidized quantitatively, but glycollic acid produced was not oxidized further. L-Tartaric acid was not oxidized even in the presence of a small amount of glucose.

Nippon Nôgeikagaku Kaishi, **35**, 122 (1961)

Hydroxylation of Steroids by *Diplodia tubericola*. Part III. Studies on the Microbiological Hydroxylation of Steroids

By Toshinobu Asai, Ko Aida, Tokuji Tanaka and Yoshihiro Sato

Division of Physiology

Studies were carried out on the microbiological transformation of various steroids by *Diplodia tubericola*. Examinations were made on the comparison of media suitable for the oxidation of progesterone, pregnenolone and Reichstein's compound S. Cultural conditions were also examined for the mass culture to decide the optimal condition. *Diplodia tubericola* was found to form 7β-hydroxyprogesterone and 7β, 15β-dihydroxyprogesterone from progesterone, 7β, 15β-dihydroxyprogesterone from 7β-hydroxyprogesterone, 7β-hydroxyprogesterone and 7β, 15β-dihydroxyprogesterone from pregnenolone, 7β-compound S and 17α, 20β, 21-trihydroxy-4-pregnen-3-one from Reichstein's compound S. The strain was also found to form succinic acid from glucose and starch.

Nippon Nôgeikagaku Kaishi, **35**, 244 (1961)

Degeneration of Ability to Sporulate in Butyric Acid Bacteria

By Seiichi Nasuno and Toshinobu Asai

Division of Physiology

A strain of butyric acid bacteria, *Clostridium butyricum* ATCC 6014, was cultivated for many generations and the degeneration of spore-forming ability and variation in fermentation products were studied.

When grown in a potato-extract medium, degeneration of spore-forming ability occurred comparatively slowly and as sugar-consuming ability gradually decreased the ratio af butyric acid formed to sugar consumed increased, and the solvent formation decreased.

As time elapsed the spore forming ability decreased and the cells became larger and longer. Especially, in a medium supplemented with 0.1% phenol the cells grew into filamentous cells with 50–100 times length of ordinary cells.

The degeneration of spore-forming ability was stimulated by acidic conditions of media and the addition of phenol, but prevented by the addition of $CaCO_3$ in the media. The inoculum size, transfer interval, and addition of NaCl had no influence on degeneration.

The degeneration phenomenon of this organism is considered to be a temporary variation caused by changes in environments.

Radioisotopes, **7**, 288–289 (1958)

Detection of phosphoric acid by the activation analysis (Preliminary report)

By Hajime Takahashi, Junko Hosoda and Hiroshi Yoshikawa

Division of Enzymology

A preliminary experiment for the detection and estimation of small amount of phosphoric acid on filter papers by an activation analysis according to the reaction $^{31}P(n, \gamma)^{32}P$ by the use of an atomic reactor JRR-1 was described. Ten to 50 μg of phosphorus was applied to various Toyo filter papers as ammonium phosphate. They were then irradiated by the reactor for 13.5 hours at 2×10^{11} n/cm^2 sec during 3 days period. After irradiation, they were cooled for 7 days, and the samples were then counted by a GM detector. Among the filter papers tested, Toyo No. 51 filter paper gave the minimum background, which was approximately 15 cpm/cm^2.

One μg of phosphorus on the filter papers gave approximately 60 cpm, thus semi-quantitative estimation of phosphorus compounds down to 0.2 μg P could be done under the conditions.

J. Biochem., **45**, (1958) 639-646

An Immunochemical Study of Microbial Amylase (I)

By Tsuru WADA and Masayasu NOMURA

Division of Enzymology

The precipitation reaction between bacterial amylase and its antibody as studied quantitatively. The molecular ratio of antibody to enzyme in the precipitate was calculated.

The enzyme-anti-enzyme neutralization reaction was found to be inhibited by substrate and antiserum on this phenomenon suggested an apparent competitive relationship between starch hydrolysate and antibody.

Various known carbohydrates were examined for their inhibitory effect on the neutralization reaction. Inhibitory effect was correlated with the structure of straight chain poly-(or oligo) saccharides containing α-glucosidic-1, 4-linkage. Inhibitory effect of starch and amylase on the amylase-anti-amylase neutralization reaction was studied kinetically. An apparent competitive relationship between substrate and antibody was also observed. The precipitation reaction between enzyme and its antibody was also found to be inhibited by its substrate (or products). It was suggested that the substrate (or products) and antibody might compete for combination in the same area of the enzyme surface.

J. Biochem., **45**, (1958) 629-637

Studies on Amylase Formation by *Bacillus subtilis*. V. Immunochemical Studies of Amylase produced by *Bacillus subtilis*

By Masayasu NOMURA and Tsuru WADA

Division of Enzymology

Antiserum produced in rabbits by the injection of crystalline amylase was found to neutralize the enzyme activity. The amount of enzyme neutralized was proportional to that of antibody added in the region of antigen excess.

Search for the protein, enzymatically inactive but capable of combining with anti-amylase neutralizing antibody, was performed both in cultural media and in cell extract. No positive result was obtained. However, in the course of these experiments, the enzymatic hydrolysate of starch was found to interfere strongly with the amylase-antiamylase reaction.

Bacillus subtilis strain N was found to produce only one and the same amylase judged from the immunochemical criterion either in the culture media or within the cell.

The effect of antibody on the amylase formation by intact cells was examined. No appreciable effect was observed.

J. Biochem., **46**, (1959) 329-336

An Immunochemical Study of Microbial Amylase (II)

By Tsuru WADA

Division of Enzymology

Antisera against Taka-α-amylase were produced in rabbits by the injection of crystalline Taka-α-amylase. Serological properties of antisera obtained were studied and it became clear that the antisera contained only a single homogeneons antibody with a moderate high activity. The inhibition of Taka-amylase activity by antibody, and the inhibitory effect of starch and other carbohydrated on the amylase-antiamylase reaction, were clearly demonstrated.

The effect of an anti-Taka-amylase antibody on the various amylases of other origin was studied. It was found that anti-Taka-amylase antiserum inhibits the α-amylase activities of Aspergillus usami, awamori and sojae; on the other hand the α-amylase activities of other molds and β-amylase activities of all strains tested were unaffected.

Heat denatured Taka-amylase did not inhibit

the reaction between the Taka-amylase and its antiserm.

J. Biochem., **45**, (1958) 737-744

Studies on Amylase Formation by *Bacillus subtilis*. VI. The Mechanism of Amylase Excretion and Cellular Structure of *Bacillus subtilis*

By Masayasu NOMURA, Junko HOSODA ane Hiroshi YOSHIKAWA

Division of Enzymology

The trigger mechanism by which the autolytic enzyme of *Bacillus subtilis* is brought into action was studied. Cyanide or a high concentration of azide which inhibited respiration of this bacterium caused lysis in aerobic condition. Anaerobic conditions also induced autolysis. Dinitrophenol did not induce but only stimulated lysis once the lysis was induced by other inhibitors. Chloramphenicol had no effect on anaerobic lysis. Thus, it was concluded that anaerobic lysis may be caused by the activation of a pre-existing autolytic enzyme and this activation may be induced by the reducing condition due to oxygen deficiency.

Lysis due to added cyanide and dinitrophenol occurred only after the culture began to produce amylase. Protoplasts seemed to be unstable during this phase. The participation of the autolytic enzyme in the process of enzyme excretion through the cell wall was proposed and discussed.

Biochim. Biophys. Acta, **29**, (1958) 161-167

Enzyme Formation in Lysozyme Lysate of *Bacillus subtilis*

By Masayasu NOMURA, Junko HOSODA and Susumu NISHIMURA

Division of Enzymology

An active lysate of *B. subtilis* was obtained by treatment with lysozyme in the presence of polyvinyl sulfate and protease inhibitor from potato extract. Such preparations retain the ability to form amylase, incorporate glycine into protein and nucleic acid, and oxidize glucose at rates comparable to those of intact cells. Such activities can be observed in the absence of osmotic regulation.

The effectiveness of the addition of polyvinyl sulfate was interpreted to be due to an inhibition of the destructive action of intracellular RNAase.

Biochim, Biophys. Acta, **31**, (1959) 125-134

Stimulation of Amylase Formation by a Ribonucleic Acid from *Bacillus subtilis*

By Masayasu NOMURA and Hiroshi YOSHIKAWA

Division of Enzymology

Further studies were performed on the nature of active factor (ribonucleic acid (RNA) preparation) which can stimulate the formation of amylase in *Bacillus subtilis*.

Ribonuclease (RNAase) treatment of the acid-precipitated active factor apparently does not destroy the stimulating effect. However, the active factor becomes acid-soluble after RNAase treatment. This indicates that active factor is RNA or substance(s) attached to RNA. In contrast to ribonuclease treatment, alkaline hydrolysis destroys activity.

The activity of RNA and its RNAase digest from various sources were examined and compared with the aciive preparation obtained from an amylase-producing culture. Yeast RNA and its RNAase digest cannot replace the active factor. Preparations derived from a culture which is very poor in amylase production are also inactive.

Specificity of action of this active factor preparation was examined. The preparation stimulates the incorporation of ^{14}C-glycine into protein to some extent. However, the same degree of stimulation is observed with RNAase digest of yeast RNA. Therefore, this stimulation can be

interpreted as being due to non-specific action of general RNA in the preparation. In contrast to incorporation, protease formation is stimulated far better by factor preparation than by RNAase digest of yeast RNA, as in the case of amylase formation.

In contrast to polymucleotide preparation reported by other workers, the active factor cannot induce amylase formation in cells which have been poor in amylase formation in a growing medium.

J. Biochem., **46**, (1959) 857-864

Studies on amylase Formation by *Bacillus subtilis*. VII. Effect of Purine, Pyrimidine and their Analogues on Exoenzyme Formation by Uracil- and Adenine-requiring Mutants

By Junko HOSODA, Masamichi KOHIYAMA and Masayasu NOMURA

Division of Enzymology

The relationship between RNA and exoenzyme formation was studied using purine- and pyrimidine-requiring mutants of *Bacillus subtilis*. These mutants produced a considerable amount of amylase and protease even in the absence of required components but the components stimulated more of less the enzyme formation. Dinitrophenol did not inhibit the enzyme formation in the absence of the required components, while it inhibited the promoting effect of the required substances.

Azaguanine inhibited the enzyme formation by uracil-requiring mutant only when uracil was present in the culture media. This result suggested that azaguanine inhibited enzyme formation by taking part in the synthesis of unphysiological nucleic acid containing azaguanine when other components of nucleic acid are available.

J. Biochem., **46**, (1959) 705-709

Effect of Fatty Acids on the Proteolysis of Proteins. II. Effect of C_8-C_{14} Fatty Acids on the Tryptic Digestion of Serum Albumin

By Michiyo KONDO

Division of Enzymology

Native horse serum albumin is remarkably more digestible with trypsin than with ovalbumin. Horse serum albumin becomes less digestible with trypsin by the addition of C_8-C_{14} fatty acids. The digestibility of serum albumin is not influenced remarkably by heating with fatty acids. The laevorotation of horse serum albumin solution decreases by the addition of fatty acid at lower concentrations but increase at higher concentrations.

J. Biochem., **46**, 161-167 (1959)
Biochim. Biophys. Acta, **30**, 430-431 (1958)
Preliminary Notes

Ribonuclease of *Bacillus subtilis*

By Susumu NISHIMURA and Masayasu NOMURA

Division of Enzymology

RNase of *B. subtilis* was accumulated in the culture medium as an exoenzyme. RNase production varied markedly according to conditions of culture, phases of growth and strains. Two types of RNases of *B. subtilis* were separated by column chromatography using the carboxylic acid cation exchange resin IRC-50 (XE-64).

By employing the acid treatment, ammonium sulfate fractionation and column chromatography, one of the enzymes was purified almost 400 fold. Its specific activity was comparable to that of crystalline pancreatic RNase. Properties of RNase were also reported.

J. Biochem., **47**, (1960) 528

An Immunochemical Study of Microbial Amylase. III.

By Tsuru Wada

Division of Enzymology

p-Phenylazobenzol Taka-amylase A is antigenic and gives rise to antisera when infected into rabbits in repeated doses. It was demonstrated from the serological studies of antisera that there is an apparent cross reaction between Taka-amylase A aed p-dhenylazobenzoyl, Taka-amylase and protease-modified p-phenylazobenzoyl Taka-amylase A.

Amylase- and maltosidase-activities of YAB-TAA and protease modified PAB-TAA were inhibited by antisera against PAB-TAA and TAA. It was found that PAB protected PTB-TAA and protease modified PAB-TAA from the inhibition by its antiserum but not protection of PAB against the neutralization by anti-TAA was observed.

The results were consistent with the suggestion of Ikenaka that both the enzymatically active sites and PAB-residue located close together on the enzyme molecule.

Biochim. Biophys. Acta, **45**, (1960) 270

Stimulation of Amylase Formation by an Amine from *Bacillus subtilis*

By Hiroshi Yoshikawa and Bunji Maruo

Division of Enzymology

An active factor which stimulates amylase formation by *Bacillus subtilis* has been extracted from *B. subtilis* has been extracted from *B. subtilis* cells and purified. The factor resembles alkyldiamines or polyalkylamines in its chromatographic behavior, its reaction with ribonucleic acids and other types of reactions.

Authentic samples of amines such as putrescine, cadaverine, spermidine and spermine have been found to stimulate amylase formation at high concentrations. None of them however is identical with the factor.

The factor also stimulates the production of other exo-enzymes of *B. subtilis*, e. g., proteases, ribonuclease, and glutamyl-transferase. Moreover it stimulate the turnover of RNA and polyphosphate in the cells to a greater extent that it stimulates enzyme production.

In vitro experiments showed that in the presence of the factor, RNA associates to larger molecules than those of the original RNA.

The chemical structure of the factor and its biological functions are discussed.

J. Biochem., **48**, (1960) 236-241

Variation of Macromolecular Components of Pseudomonas-P at Different Cultural Age

By Hiromi Mitsui, Yoshiharu Maruyama and Etsuko Yoshida

Division of Enzymology

Electrophoretic analysis of the sonic extracts of *Pseudomonas*-P revealed that they were composed of at least 7 components. Each component was separated by zone electrophoresis and characterized by chemical analysis and spectrophotometry. These components were: free DNA, RNA, ribonucleoprotein, cytochrome rich fraction and protein accompanied with polysaccharide.

It was found that the content of ribonucleoprotein was higher in the early log-phase cells than in the stationary-phase cells. On the other hand, the amount of the cytochrome rich fraction was greater than that of the stationary-phase cells.

Streptomycin precipitated all of the nucleic acids containing fractions under the specified condition.

The variation of cellular components was discussed from the physiological view point.

J. Biochem., **48**, (1960) 242-250

Incorporation of Amino Acids into Protein of Pseudomonas-Cell Fractions

By Hiromi MITSUI and Etsuko YOSHIDA

Division of Enzymology

Cell fractions were prepared from *Psudomonas-P* by sonic disintegration and differential centrifugation. These fractions were composed of large particles, sedimentable at low speed centrifugation ($20,000 \times g$), small particles, sedimented at $105,000 \times g$, and the soluble fraction. Chemical, ultracentrifugal and electrophoretic analysis gave some characteristics of each fraction.

Both *in vivo* and *in vitro* experiments showed that the rate of amino acid incorporation into particulate fractions was much higher than in the soluble fraction. The activity of the particulate fraction was markedly inhibited by the soluble fraction.

A 'Ghost' fraction and a cytoplasmic supernate were obtained by lysozyme treatment in hypertonic medium and subsequent osmotic shock of the protoplasts. These two fractions incorporated amino acid at the same rate *in vivo*. The role of the particulate fraction of *Pseudomonas* cells was discussed.

J. Biochem., **48**, (1960) 521-261

Amino Acid Incorporation by a Bacterial Cell-Free System

By Etsuko YOSHIDA, Hiromi MITSUI, Hajime TAKAHASHI and Bunji MARUO

Division of Enzymology

Preparation of subcellular fraction from *Pseudomonas-P* and their activities of amino acid incorporation were investigated.

Concentration of $MgSO_4$ in the resuspending medium was closely related to the activity of the preparation. Optimal concentration of magnesium ion was 10^{-2} M, and the preparation lost its activity at the concentration of less than 10^{-4} M. A large amount of RNA and protein were released from the precipitate fraction (MgP) in the absence of magnesium ion.

Amino acid incorporation occurred only under the aerobic condition. The behaviour against various inhibitors suggested that the amino acid incorporation was closely related to the oxidative phosphorylation in this system.

The effect of magnesium ion on both the ribonucleoprotein particles and this system was discussed.

Biochim. Biophys. Acta, **45**, 15-27 (1960)

Extracellular Ribonucleases from *Bacillus subtilis* I. Crystallization and Specificity

By Susumu NISHIMURA

Division of Enzymology

A method was described for large scale purification of two extracellular RNases obtained from *Bacillus subtilis*. They were here named RNase peak I and RNase peak II respectively. RNase peak II was crystallised in the form of needles from homogeneous preparations obtained by the use of chromatography.

The specificity of the RNase peak I and peak II enzymes was quite different from that of bovine pancreatic RNase. They hydrolyzed the "core" remaining after treatment of the yeast RNA fraction with bovine pancreatic RNase. But they dydrolyzed only the secondary phosphate esters of the purine riboside 3'-phosphates in RNA, and cyclic gnanylic and adenylic acids were then isolated as intermediary products from the dialysate. The Mononucleotides and end groups of the oligonucleotides produced by the enzymes were guanylic and adenylic acids.

The effects of metallic salts, polyvinyl sulfate and anti-RNase on the activity of RNase peak I and RNase peak II and bovine pancreatic RNase are described.

A protein, which has no RNase activity, was obtained in crystalline form by crystallisation of

the fraction of peak I, and the sedimentation coefficient of this inactive protein was about the same as that of active RNase peak II.

A latent RNase, which was activated by 4 M urea and was almost completely inhibited by anti-RNases, was detected in the bacterial cells.

Biochim. Biophys. Acta, **40**, 355-357 (1960

Intracellular Ribonuclease from *Bacillus subtilis*

By Susumu Nishimura and Bunji Maruo

Division of Enzymology

The presence of an intracellular RNase in *Bacillus subtilis* which had quite different properties from the extracellular RNases could be detected. A small amount of RNase activity which was shown in the lysozyme lysate was not inhibited at all by the addition of anti-extracellular RNases which had been shown substantially to inhibit extracellular RNases.

Intracellular RNase in the lysate was precipitated at pH 4, and completely inactivated by heating at 100° for 5 min. The pH optimum of the enzyme was about 5.8, whereas that of the extracellular RNases were 7.5. The intracellular RNase was completely inhibited by the addition of 1.7×10^{-3} M EDTA, although the same concentration of EDTA had no effect on extracellular RNases.

The intracellular RNase was purified about 17 times and could be separated from the phosphodiesterase activity by the method of ammonium sulfate fractionation, streptomycin treatment and starch zone electrophoresis.

Purified RNase produced all four constituent mononucleotides from yeast RNA. Nucleosides or free bases could not be detected. All four cyclic mononucleotides could be isolated as intermediary products from the dialysate.

The presence and distribution of two types of RNases, i.e. intracellular acid RNase and extracellular alkaline RNase, in this organism seems to be different from the two RNases described for mammalian tissues.

J. Biochem., **49**, (1961) 411-413

Incorporation of Amino Acids into Cytochrome c in a Bacterial Cell-Free System

By Hiromi Mitsui

Division of Enzymology

A cell-free preparation from crushed protoplasts of Pseudomonas-P incorporated C^{14}-labeled amino acids actively into protein fraction.

C^{14}-labeled cytochrome c_{554} was isolated and characterized from the reaction mixture.

J. Agr. Chem. Soc. Japan, **34**, (1960) 975-977

Tritium Counting with a Windowless Gas Flow Counter

By Shigeki Mizuno, Hajime Takahashi and Bunji Maruo

Division of Enzymology

The method was described to determine the radioactivity of ^{3}H-compounds at infinite thickness by using a filter paper-disk. Tritiated sample was put on the filter paper-disk (1.9 cm diameter), placed in the centre of an iron planchet (5 cm^2) and was counted with a windowless gas flow counter after drying under an infrared lamp. Using this paper-disk method good proportionality between the total radioactivity of added ^{3}H-adenosine (0–1.0 mg/cm^2, 0.3 ml/disk) the actual count was demonstrated.

Counting error was within $\pm10\%$, counting efficiency was about 0.3% of the total disintegration events.

J. Antibiot. Ser. A, **12**(5), 195-200 (1959)

Studies on Variotin, A New Antifungal Antibiotic. I. Preparations and properties of variotin

By Setsuo TAKEUCHI, Hiroshi YONEHARA and Hamao UMEZAWA

Division of Antibiotics

A new antifungal antibiotic was isolated from the culture filtrates of *Paecilomyces varioti var. antibioticus*. The antibiotic was named variotin. Variotin is a colorless oily substance and soluble in many organic solvents except petroleum ether and water.

The formula $C_{18}H_{27}O_4N$ was given to variotin. Variotin shows activities against various kinds of fungi and has low toxicity on mice.

J. Antibiot. Ser. A, **12**(5), 201-202 (1959)

Studies on Variotin, A New Antifungal Antibiotic. II. Taxonomical studies on variotin-producing strain

By Shigeo ABE, Setsuo TAKEUCHI and Hiroshi YONEHARA

Division of Antibiotics

Morphologically, the variotin-producing strain is considered to belong to *Paecilomyces varioti* Bainier. However, the strain differs from the descriptions on *Paecilomyces varioti* Bainier litter by Raper and Thom or by Brown and Smith, primarily in the typically funiculose texture, olivaceus black shade on revers, uncolor or occationally bluish gray green colored pigment in surrounding agar, and the production of a antifungal antibiotic.

Careful consideration of all described species and variaties of *Paecilomyces* has failed to identify the variotin-producing strain.

The authors, therefore, present a new variety to which the name *Paecilomyces varioti* Bainier *var. antibioticus* is applied.

J. Antibiot. Ser. A, **14**(1), 44-53 (1961)

Studies on Variotin, A New Antifungal Antibiotic. III. Chemical structure of variotin

By Setsuo TAKEUCHI and Hiroshi YONEHARA

Division of Antibiotics

Variotin is a new antifungal antibiotic, it is neutral oily substance, having a molecular formula of $C_{18}H_{27}O_4N$. Its ultraviolet absorption spectrum showed maximum at 320 mμ. The variotin cosumed 4 moles of hydrogen by catalytic hydrogenation. The hydrogenated product of variotin had a molecular formula of $C_{18}H_{35}O_4N$ and was designated as octahydrovariotin.

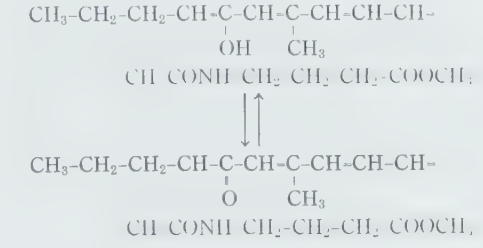
On hydrolysis of octahydrovariotin with N/2 sodium hydroxide at room temperature gave an acidic substance named octahydrovariotic acid ($C_{17}H_{33}O_3N$). The methylation of octahydrovariotic acid with diazomethane yielded the original octahydrovariotin again, evidencing that the original variotin is a methyl-ester.

Hydrolysis of octahydrovariotic acid with N/2 sodium hydroxide under reflux, gave γ-aminobutyric acid and C_{13}-hydroxy acid ($C_{13}H_{26}O_3$).

Hydrolysis of octahydrovariotin with 6N hydrochloric acid also yielded γ-aminobutyric acid and lactone from of C_{13}-hydroxy acid.

Oxitative degradation of C_{13}-hydroxy acid with concentrated nitric acid gave two volatile fatty acids (n-butyric acid and n-valeric acid) and two dicarboxyric acids (α-methylpimetic acid and β-metylsubecic acid). Thus, the authors present the chemical structure of octahydrovariotin, methyl N-(6-methyl-8-hydroxy-dodecanoyl)-piperidata.

Chemical structure of variotin.



From the observation of ozonolysis and ultraviolet and infrared absorption spectrum, the authors present the chemical structure for variotin as methyl N-(6-methyl-8-hydroxy-dodeca-2, 4, 6, 8-tetraenoyl)-piperidate or methyl N-(8-oxo-6-methyl-dodeca-2, 4, 6-trienoyl)-piperidate.

J. Antibiotics Ser. A, **13**, (1960) 401-405

On a new antibiotic, emimycin

By Michiya Terao, Keiko Karasawa, Nubuo Tanaka, Hiroshi Yonehara and Hamao Umezawa

Division of Antibiotics

Emimycin is an antibiotic produced by a streptomycete isolated from a soil sample, *Streptomyces* No. 2020-I, which resembles to *Streptomyces antibioticus* and *Streptomyces griseochromogens*, in a medium containing starch, sucrose, soybean meal, dry yeast and $CaCO_3$.

The antibiotic produced was isolated by adsorption processes using active carbon and anion exchange resin of hydroxyl type. It was recrystallized from hot methanol and obtained as white needle crystals posessing the empirical formula of $C_8H_8N_4O_4$. The physical and chemical properties indicate that the antibiotic is new and named emimycin.

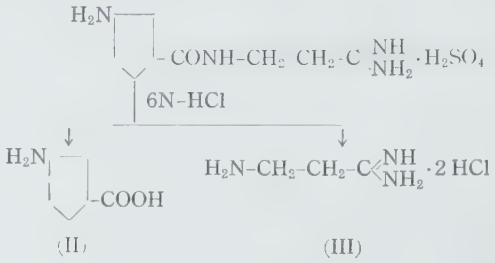
It exhibits partial inhibition against various species of bacteria and some yeasts along with is low toxicity.

J. of Antibiotics Ser. A, **12**(5), 326 (1960)

Structure of Amidinomycin

By Shoshirō Nakamura, Keiko Karasawa, Nobuo Tanaka, Hiroshi Yonehara and Hamao Umezawa

The chemical structure of amidinomycin was studied. Acid hydrolysis of amidinomycin gave two ninhydrin-positive compounds, 3-amino-1-carboxycyclopentane (II) and 2-amidinoethylamine (III), and the structure of the antibiotic, N-(2'-amidinoethyl)-3-aminocyclo-pentanecarboxamide (I) was determined. Isolation of 3-amino-1-carboxy-cyclopentane is the first example from natural souces.



J. Antibiotics Ser. A, **12**, 86-89 (1959)

Studies on Mikamycin. III

By Koichi Okabe

Division of Antibiotics

Mikamycin was crystallized from ethylacetate eluate of alumina chromatography. Monoacetate, di-2, 4-dinitrophenyl-hydrazone, decahydro derivertives of mikamycin were prepared. From the elementary analysis and the molecular weight determination of the above derivatives, the molecular formula of mikamycin previously proposed, $C_{31}H_{39}O_6N_3$ was reconfirmed. It was determined that mikymycin had 2 C-CH_3, 1 N-CH_3, 1, acetylable OH, 2 CO and 5 C=C. 1-Proline and glycine were isolated from the hydrolysate.

J. Antibiotics Ser. A, **12**, 192-193 (1959)

Alkaline hydrolysis of mikamycin A

By Koichi Okabe, Hiroshi Yonehara and Hamao Umezawa

Division of Antibiotics

Mikamycin A, $C_{31}H_{39}O_9N_3$, was hydrolyzed in 0.5 N sodium hydroxide-methanol solution and a yellow granular crystals, named mikamycinine,

mp. 258-260 and a carboxlic acid, named mikaic acid, bp. 83.5 were obtained.

J. Antibiot. Ser. A, **13**, 410-411 (1960)

Structure of keto acid obtained by degradation of mikamycin A

By Koichi OKABE
Division of Antibiotics

Mikamycinine was hydrolyzed in 10% sodium hydroxide solution for 24 hours under reflux. The saponified solution was acidified with diluted sulfuric acid to pH 2.0 and steam distilled. A keto acid, $C_{13}H_{18}O_3$, b.p. 89°/1 mm Hg was obtained as a oil.

From the results of ozonolysis of the keto acid, oxidation of hexahydro-keto acid and the spectrometric observations of these acids, the structure, 2, 10-dimethyl-5-oxo-undeca-3, 6, 8-trienic acid, was proposed for the keto acid.

J. Antibiot. Ser. A, **13**, 412-414 (1960)

Structure of Mikamycinine, a main hydrolysis product of mikamycin A

By Koichi OKABE
Division of Antibiotics

Mikamycinine $C_{22}H_{32}O_7N_2$ is a main hydrolysis product of mikamycin A. It was hydrolyzed in concentrated hydrochloric acid alcohlic solution for 10 hours under reflux. The hydrolysate was steam distilled, and from distillate a keto oxy acid, $C_{13}H_{20}O_4$ was obtained. The keto oxy acid turned into the keto acid by boiling with 10% sodium hydroxide solution. The structure, 2, 10-dimethyl-3-hydroxy-5-oxo undeca-7, 9-dienoic acid was proposed for the keto oxy acid. Mikamycin A was reduced by Meerwein-Ponndorf reduction and the reduced mikamycin A was hydrolyzed by 6 N hydrochloric acid. The hydrolysate contained D-proline, L-threonine, trace of glycine and another ninhydrin-positive substance, indicating the existence of residue of 1-amino-2-oxo-butyric acid in mikamycin A. Hydrazinolysis of mikamycinine gave free proline confirming C-terminal to be proline. Thus, the structure, N-(2'10'-dimethyl-3'-hydroxy-5'-oxo-undeca-7', 9'-dienoyl)-L-1-amino-2-oxo-butyryl-D-proline was proposed for mikamycinine.

J. Antibiot. Ser. A, **13**, 57-61 (1960)

Studies on Mikamycin. IV. The isolation and properties of mikamycin B

By Kiyoshi WATANABE
Division of Antibiotics

In the course of production studies of mikamycin, another antibiotic of different nature was isolated and designated mikamycin B. Consequently the previous one was named mikamycin A. The distinct synergistic relation was observed between these two mikamycins A and B. The production process of mikamycins and purification procedure of mikamycin B from crude substances are described, as well as the physical and chemical properties of it.

J. Antibiot. Ser. A, **13**, 62-69 (1960)

Studies on Mikamycin. V. In vitro synergistic action and differential assay of mikamycin components

By Kiyoshi WATANABE
Division of Antibiotics

The synergistic relation of various combinations of mikamycin A and B were investigated in liquid dilution method against Staphylococcus aureus 209 p, as well as in cylinder plate method against three gram-positive organisms. The principles of the differential assay methods of two synergistic components were described and the most appropriate method was presented.

J. Antibiot. Ser. A, **14**, 1-13 (1961)

Studies on Mikamycin. VI. Constitutive amino acids of mykamycin. B

By Kiyoshi Watanabe
Division of Antibiotics

Mikamycin B was found to constitute the following amino acids: 3-hydroxypicolinic acid, L-threonine, D-α-amino-n-butyric acid, L-proline, L-phenylglycine, L-4-oxo-pipecolinic acid and L-p-dimethyl-amino-N-methyl-phenylalanine, the last of which had not been encountered in natural kingdom nor synthesized yet.

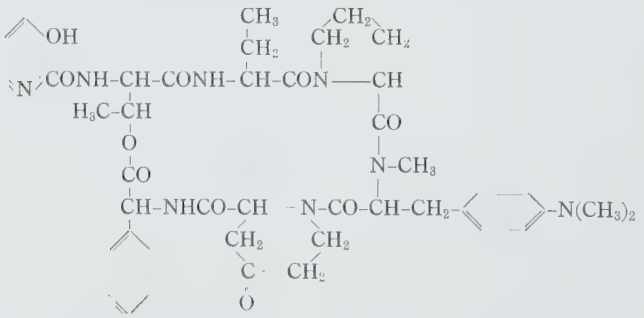
J. Antibiot. Ser. A, **14**, 14-17 (1961)

Studies on Mikamycin. VII. Structure of mikamycin B

By Kiyoshi Watanabe
Division of Antibiotics

Hydrazinolysis of mikamycin B produced no C-terminal amino acid nor acid hydrolysis of DNP-mikamycin gave any N-DNP amino acid except O-DNP hydroxypicolinic acid. Mikamycin B-acid obtained by alkaline hydrolysis showed phenolic group and a new carboxyl group. This acid still contained all the seven constituent amino acids, this opening of the lactone in mikamycin B was suggested.

Hydrazinolysis of mikamycin B-acid proved phenylglycine to be C-terminal amino acid.



From various acid hydrolysate, 3 fluorescent peptides were isolated, amino acid sequences in them were as the following: Hydroxypicolinyl-threonine (I), hydroxypicolinyl-threonyl-α-amino-n-butyric acid (II), hydroxypicolinyl-threonyl-α-amino-n-butyryl-prolyl-p-dimethylamino-N-methyl-phenylalanine (III). From these results, the following structure was proposed for mikamycin B.

J. Antibiotics Ser. A, **11**, 127-133 (1958)

Biological studies on mikamycin

By Nobuo Tanaka, Nagashige Shinjo, Norimasa Miyairi and Hamao Umezawa
Division of Antibiotics

A newly isolated antibiotic of low toxicity, designated as mikamycin, has been evaluated by *in vitro* and *in vivo* laboratory studies. It is inhibitory against various genera and species of gram-positive organisms *in vitro*. In spite of the low antibacterial activity of the blood and tissues after the administration of mikamycin, it is able to produce a pronounced therapeutic

effect in the mouse protection tests against pyogenic streptococcus and staphylococcus infections. The mechanism involved in this discrepancy is discussed.

J. Antibiotics Ser. A, **12**, 290-297 (1959)

Biological studies on mikamycin. II. Laboratory investigations of mikamycin A and mikamycin B

By Nobuo TANAKA, Norimasa MIYAIRI Kiyoshi WATANABE, Toshio NISHIMURA, Nagashige SHINJO and Hamao UMEZAWA

Division of Antibiotics

The biological activity of two constituents A and B of mikamycin was studied. Both are inhibitory against various genera and species of gram-positive microbes *in vitro*, though the antimicrobial spectrum are slightly different. A marked synergistic activity of A and B is observed both *in vitro* and *in vivo*. Blood levels of both antibiotics are low, when injected either singly or in the form of mixture. In contrast to the low antibacterial activity in blood, a marked protection of mice infected with hemolytic streptococci and pneumococci is demonstrated. These antibiotics are of low toxicity.

J. Antibiotics Ser. A, **14**, 18-22 (1961)

Activity of mikamycins, angustmycins and emimycin against antibiotic-resistant staphylococci

By Nobuo TANAKA, Norimasa MIYAIRI, Toshio NISHIMURA and Hamao UMEZAWA

Division of Antibiotics

The *in vitro* activity of mikamycins, emimycin and angustmycins was investigated on antibiotic-resistant staphylococci, both laboratory-developed strains and clinical isolates. They showed no cross resistance with penicillin, streptomycin, chloramphenicol, tetracycline, streptothricin, telomycin, amphomycin, erythromycin and carbomycin. The marked difference in the sensitivity to angustmycins among the strains of *Staphylococcus aureus* indicates the necessity of using a variety of strains for screening tumor-inhibitory antimetabolic antibiotics.

J. Antibiotics Ser. A, **14**, 60 (1961)

Mechanism of action of mikamycin

Nobuo TANAKA, Hideyo YAMAGUCHI and Hamao UMEZAWA

Division of Antibiotics

In the studies of the mode of action of mikamycin, it was found that mikamycin A quantitatively inhibits the incorporation of ^{14}C-amino acids into the hot acid-insoluble and trypsin-digestible fraction of the organism. And the depression in ^{14}C-amino acids incorporation appears to be parallel to the bacteriostatic activity. Mikamycin B does not significantly influence the incorporation. The same synergism of A and B is observed in the inhibition of ^{14}C-amino acids incorporation, as demonstrated in the case of the bacteriostatic activity. The same inhibitory effects are found in ^{14}C-glutamate incorporation into hot acid-insoluble and trypsin-digestible fraction (protein fraction), but not into hot acid-insoluble and trypsin-indigestible fraction (cell wall fraction). In this sense, the activity of mikamycin A is similar to that of chloramphenicol, but not of penicillin. The results indicate that mikamycin interferes with protein synthesis and that this effect may be connected with the bacteriostatic activity.

J. Antibiotics Ser. A, **13**, 265-269 (1960)

Studies on antagonists to angustmycins

By Nobuo TANAKA, Norimasa MIYAIRI and Hamao UMEZAWA

Division of Antibiotics

In vitro antibacterial activity of angustmycins

A and C were investigated. They were observed to be powerful antagonists to nucleosides and purines in microbial growth. The activities were specifically reversed by guanine, guanosine, adenosine, xanthosine and inosine. Mechanism of action of angustmycins was discussed.

J. Antibiotics Ser. A, **14**, 23-26 (1961)

Differential bioassay of angustmycins A and C

By Nobuo Tanaka, Norimasa Miyairi and Hamao Umezawa

Division of Antibiotics

Antimicrobial spectrums of angustmycins A and C are similar, and differential bioassay cannot be performed by using ordinary strains. However, in the course of study on antibiotic-resistant staphylococci, a couple of clinical isolates, R 6 and R 7, resistant to penicillin and tetracycline, were found to be sensitive to C but resistant to A. This observation led us to a study of differential assay procedure of angustmycins. Assay of angustmycin C in mixtures of A and C is performed in a semisynthetic medium supplemented with casamino acids, using *Staphylococcus aureus* R 7 as a test organism. Assay of angustmycin A in mixtures of both antibiotics is carried out in a synthetic medium, using *Mycobacterium sp. 607* as a test organism.

J. Antibiotics Ser. A, **14**, 98-102 (1961)

Activity of angustmycins against experimental infections and transplantable tumors

By Nobuo Tanaka, Toshio Nishimura Hideyo Yamaguchi and Hamao Umezawa

Division of Antibiotics

The *in vivo* antibacterial and antitumor effects of angustmycins A and C were investigated. The results fluctuated from one experiment to another, which appeared to be due to the antimetabolic nature of the antibiotics. Both substances exhibited considerable effects against staphylococcal and streptococcal infections in mice. Angustmycin C was more active than angustmycin A *in vivo* against the gram-positive organisms, which is in accordance with the *in vitro* activity. They showed considerable activity against Walker adenocarcinoma 256 in rats, and much less activity against adenocarcinoma 755, Ehrlich carcinoma and sarcoma 180 of solid forms in mice. By the method employed, no tumor inhibition was observed with ascitic tumors, including Yoshida sarcoma, Ehrlich carcinoma and sarcoma 180.

Japan. J. Microbiology, **3**, 191-201 (1959)

Histochemical studies on the cellular distribution of endotoxin of Salmonella enteritidis in mouse tissues

By Nobuo Tanaka, Toshio Nishimura and Toshihisa Yoshiyuki

Division of Antibiotics

The endotoxin of *Salmonella enteritidis* was prepared and characterized by biological and chemical methods. The fate of the endotoxin in mouse tissues was studied histochemically, using fluorescein-labeled antibody technique. The most prominent area where endotoxin was localized was in the cells of the reticuloendothelial system, particularly the Kupffer cells of the liver and the large round cells of the splenic red pulp. In addition, the endotoxin was detected in the vessel endothelium of various organs. The distribution of the endotoxin was limited to the cytoplasm, but not to the nucleus. Studies on the persistence of the endotoxin in the tissues were performed with an intravenous injection of 0.5 mg per mouse. It remained for approximately 12 weeks in the macrophages of the liver. The relationship of the localization of the endotoxin to the biological activity, including Shwartzman phenomenon and tumor-damaging effects, was

discussed.

Japan. J. Microbiology, **3**, 267–276 (1959)

Histochemical studies on experimental typhoid by means of fluorescein-labeled antibody. I. Cellular localization of the antigens of Salmonella enteritidis after injection of the killed vaccines

By Nobuo TANAKA, Toshio NISHIMURA and Toshihisa YOSHIYUKI

Division of Antibiotics

The cellular localization of the antigens of *Salmonella enteritidis* in the mouse tissues after intravenous and intraperitoneal injections of the killed organisms (heat-killed and chrome-vaccine) was immunohistochemically investigated. In the liver, the antigens were observed to fill the cytoplasms of the Kupffer cells and those of the leukocytes infiltrated around the sinusoids and other vessels. When the granulomas were formed, the antigens were demonstrated in their macrophages in various amounts. In the spleen, the antigens were localized in the cytoplasms of the macrophages of the red pulp. Biological significance of the distribution of the antigens was discussed.

Japan. J. Microbiology, **4**, 35–42 (1960)

Histochemical studies on experimental typhoid by means of fluorescein-labeled antibody. II. Cellular localization of the antigens of Salmonella enteritidis in the process of the infection of experimental typhoid

By Nobuo TANAKA, Toshio NISHIMURA and Toshihisa YOSHIYUKI

Division of Antibiotics

In the process of infection of experimental typhoid, histological observation of the causative organisms in the liver and spleen is negative, despite the fact that a number of living organisms are demonstrated by bacteriological culture technique. The histochemical follow-ups of bacterial cells of *Salmonella enteritidis* (rod-shaped antigens) in the mouse tissues were successful by fluorescent antibody techuique. The antigens were also observed in the specific granulomas (typhomas), which were formed in the liver and spleen. It was established by this study that fluorescent antibody is a useful tool of demonstrating the parasites in the host, which cannot be observed by conventional staining technique.

Japan. J. Microbiology, **4**, 433–449 (1960)

Histochemical studies on experimental typhoid by means of fluorescein-labeled antibody. III. Demonstration of gamma globulin or antibody in the typhoid granuloma

By Nobuo TANAKA, Hideyo YAMAGUCHI, Toshio NISHIMURA and Toshihisa YOSHIYUKI

Division of Antibiotics

Homologous γ-globulin has been successfully demonstrated in the typhoid granuloma of mouse liver and spleen following injections of live organism or killed vaccine of a virulent strain of *S. enteritidis*. It is established by the antigen inhibition test that γ-globulin in the granuloma is the specific antibody. The observations suggest that antibody is produced by these macrophages. Another prominent locus of γ-globulin is observed in cells of the plasma cell series, including Russell bodies. Small amounts of γ-globulin are detected in the lymphocytes. Antibody is found chiefly in the cytoplasm, but occasionally it is located in the nucleus of these cells including the granuloma macrophages. It is indicated that most of serum antibody is produced by cells of the plasma cell series; and a portion by the granuloma macrophages and lymphocytes. The present study presents a new

type of antibody-synthesizing apparatus: i.e. the granuloma macrophages.

Japan. J. Microbiology, **3**, 345-353 (1959)

Histochemical studies on Newcastle disease virus (NDV) by fluorescent antibody technique. I. The virus antigen formation in cultured human cells

By Nobuo Tanaka, Hideyo Yamaguchi and Masami Kobayashi

Division of Antibiotics

The successful demonstration of NDV multiplication in HeLa cells by hemagglutination and fluorescent antibody technique was presented. The first appearance of the virus antigen (at 12 hour) was limited to the perinuclear region of the cytoplasm. The number and density of the antigen particles were increased with time. They fused to each other, some of them occurred in the other parts of the cytoplasm. In most of the cells, the next outstanding localization of the antigen was found in the surface region of the cells. It occurred in the form of a band and was getting denser and thicker. At this stage the virus seemed to be released into the medium from the surface of the cells, judging from the growth curve of virus. In some of the cells, however, another process of virus antigen accumulation took place in the later period. At 48 to 72 hours, large and dense granules of the virus appeared in the cytoplasm. Their number was increased, the virus antigen filled the most part of the cytoplasm, and finally it caused the lysis. The latter appearance of the virus antigen might be due to phagocytosis of the virus by the cells. Binuclear and multiple-nuclear cells were observed in virus-containing cells. In indicated that the mitosis or division occurred in virus-multiplying cells.

Nature, **188**, 873-875 (1960)

Knall-gas Reaction-linked Fixation of Labelled Carbon Dioxide in an Autotrophic *Streptomyces*

By Ryuzi Kanai, Shigetoh Miyachi and Atusi Takamiya

Division of Biosynthesis I

It was found that autotrophic *Streptomyces* was capable of fixing carbon dioxide by using the energy derived from the oxyhydrogen reaction. It was also shown that the capacity of this organism for fixing carbon dioxide was greatly increased when the cells had been allowed to carry out the oxyhydrogen reaction in the absence of CO_2.

Plant and Cell Physiol., **1**, 151-153 (1960)

Effect of CO_2 on the Levels of DPNH and TPNH in Pre-Illuminated *Chlorella* Cells

By Shigetoh Miyachi, Tamiko Oh-hama and Hiroshi Tamiya

Division of Biosynthesis I

Using intact cells of *Chlorella*, investigations were made on the effect of CO_2 on the levels of DPNH, TPNH and the photogenic reducing agent (R) in pre-illuminated cells. On turning off the light, R rapidly decreased to the zero level, and the rate of the decrease was markedly increased by the presence of CO_2. On the other hand, both TPNH and DPNH behaved quite differently, showing irrespective of the presence or absence of CO_2, a temporary increase followed by a gradual decrease. These results may be taken as an additional evidence for our earlier conclusion that R can be neither TPNH nor DPNH.

Plant and Cell Physiol., **1**, 155-162 (1960)

Changes in Levels of Pyridine Nucleotides in *Chlorella* Cells during the Course of Photosynthesis

By Tamiko OH-HAMA and Shigetoh MIYACHI

Division of Biosynthesis I

In interpreting the role of pyridine nucleotide in photosynthesis, the present study was designed to follow the light-induced changes in DPN- and TPN-levels as influenced by the presence of carbon dioxide in the reaction medium. From the experimental results thus obtained, it was concluded that TPNH is the sole form of pyridine nucleotide which shows a decrement concomitantly with the progress of photosynthetic carbon fixation in intact cells of *Chlorella*.

Discussions were made on the difference in the sites of participation of TPNH and of the photogenic reducing agent in the path of carbon in photosynthesis.

Biochim. Biophys. Acta, **34**, 202-210 (1959)

Effects of Illumination and Oxygen Supply upon the Levels of Pyridine Nucleotides in *Chlorella* Cells

By Tamiko OH-HAMA and Shigetoh MIYACHI

Division of Biosynthesis I

1. Using intact cells of *Chlorella*, investigations were made on the effect of light on the levels of various forms of pyridine nucleotides, compared with that on the photogenic reducing agent (*R*).

2. No form of pyridine nucleotide was found of which the concentration was affected in the same manner as that of *R*, which has been known to be produced only in the light and eliminated completely in the dark.

3. Under both aerobic and anaerobic conditions, light decreased the level of DPN^+ and increased the level of TPN. Quantitatively, there was a correspondence between these effects.

4. When algal cells were transferred in the dark from anaerobic to aerobic conditions, a marked decrease of DPNH occurred, the levels of DPN^+, TPN^+ and TPNH remaining almost unchanged. The decrease of DPNH in the light (in N_2) was assumed to be brought about by the O_2 produced concomitantly with the photogenic formation of *R* and effecting the transfer of DPNH to some substance other than DPN^+.

5. It was concluded that (a) neither TPNH nor DPNH is the primary *R* and that (b) one of the effects caused by light is the conversion of DPN^+ to TPN.

Plant and Cell Physiol., **1**, 117-130 (1960)

Effect of Some Sulfhydryl Reagents on Light-Induced CO_2-Fixation in *Chlorella*

By Shigetoh MIYACHI

Division of Biosynthesis I

1. Using intact cells of *Chlorella ellipsoidea*, investigations were made on the effects of some SH-reagents (IAA, CMB and arsenite) upon various reactions pertaining to the primary photogenic agent (designated by *R*) formed in the mechanism of photosynthesis.

2. The pre-illumination experiments using $^{14}CO_2$ as a tracer have led us to the inference that (i) the process of photochemical formation of *R* is inhibited by IAA, that (ii) the spontaneous decay of *R* in the dark is markedly accelerated by CMB and arsenite, but not at all affected by IAA, and that (iii) the participation of *R* in the cyclic path of carbon leading to the fixation of CO_2 is not affected by IAA and CMB.

3. Based on the assumption that *R* is a reducing agent, it was discussed that the fact mentioned under (iii) is incompatible with the idea that the reduction of PGA to triose phosphate be the sole or rate-determining reductive step in the cyclic path of carbon in photosynthesis.

4. The possibility that *R* may have a functional SH-group(s) was invoked to account for the observation that the decay of *R* in the dark was markedly accelerated by CMB and arsenite.

Plant and Cell Physiol., **1**, 1-15 (1959)

Effect of Poisons upon the Mechanism of Photosynthesis as Studied by the Pre-illumination Experiments Using Carbon-14 as a Tracer

By Shigetoh Miyachi

Division of Biosynthesis I

1. Using *Chlorella* as the material, and ^{14}C as a tracer, kinetic studies were performed on the fate of the photogenic agent (denoted by *R*) which have been detected by Calvin and Benson in their "pre-illumination experiment".

2. The processes of photochemical formation and non-photochemical decay of *R*, as well as the process of $^{14}CO_2$-fixation caused by *R* were found to be affected in different manners by cyanide and azide.

3. Based on the theory propounded earlier by the author and his coworkers, the various phenomena occurring in the presence and absence of the poisons were analyzed kinetically. Quantitative data were presented in confirmation of the earlier observations that at 25° cyanide inhibits the reaction between *R* and CO_2 and accelerates the decay of *R*, while azide inhibits the reaction between *R* and CO_2, leaving the rate of decay of *R* apparently unchanged.

Biochim. Biophys. Acta, **35**, 180-189 (1959)

Role of Sulfur in the Cell Division of *Chlorella*, Studied by the Technique of Synchronous Culture

By Eiji Hase, Hama Otsuka, Sayoko Mihara and Hiroshi Tamiya

Division of Biosynthesis I

1. *Chlorella* was grown synchronously under controlled supply of S- and N-sources so as to make the processes of nuclear and cellular division proceed stepwise, and by using ^{35}S as a tracer the fates of S-compounds in various fractions of cell material (extracts with 70% ethanol, with 10% trichloroacetic acid, and the residue) were followed.

2. In the cells grown in the normal medium, the S-content (in %) of the ethanol- and TCA-extracts decreased in the growing stage and increased markedly at the ripening stage.

3. The ethanol extract contained an unknown S-compound and a non-S-containing peptide-like substance, the latter substance appearing only at certain stages of cell ripening. When chromatographed on paper the TCA extract gave only 1 radio-active (but ninhydrin-negative) spot, which on hydrolysis with HCl, turned ninhydrin-positive owing to the liberation of several amino acids including cyst(e)ine.

4. In the cells, whose nuclear and cellular division were controlled by limited supply of S- and N-sources, the quantity of the S-containing substance in the TCA-extract increased markedly before nuclear division, and the unknown non-S-containing substance in the ethanol extract appeared only before and concomitantly with, the occurrence of cellular division.

Arch. Biochem. Biophys., **83**, 170-177 (1959)

Sulfur-Containing Peptide-Nucleotide Complex Isolated from *Chlorella* and Yeast Cells

By Eiji Hase, Sayoko Mihara, Hama Otsuka and Hiroshi Tamiya

Division of Biosynthesis I

1. From the cells of a green alga (*Chlorella*) and yeasts (*Saccharomyces formosensis* and *S. cerevisiae*), a new sulfur-containing peptide-nucleotide complex was obtained by extracting the cells with cold trichloroacetic acid (10%). The complex was revealed to contain cyst(e)ine as the major sulfur component in the peptide moiety, and adenine and uracil as the major bases in the nucleotide moiety.

2. In the field of zone electrophoresis, the complex as a whole moved, unlike free nucleotides and polynucleotides, toward the cathode at pH 3.6 in the case of *Chlorella* and at pH 3.6-5.0 in

the case of yeast. At pH 5.0, the complex obtained from *Chlorella* split into a number of components, the majority of which moved toward the anode. The unsplit complex obtained at pH 3.6 gave, on hydrolysis followed by paper chromatography, several unidentified components, besides adenine, uracil, and a number of amino acids. Some of these unidentified components gave a red color with ninhydrin reagent, and they were assumed to be the factors making the complex assume a positive charge as a whole.

3. The complex obtained at pH 3.6 from *Chlorella* was also split into a number of subunits when it was subjected to an anion-exchange chromatography (Dowex 1). The complex has, thus, a property of being readily disintegrated by changes of pH or other conditions into its components different degrees of complexity.

onset of cell division, a sulfur-containing peptide-nucleotide compound(s) (SPN), which has been reported earlier, appeared in a large quantity in the cold PCA-soluble fraction, and that its quantity decreased gradually during the subsequent process of cell division, suggesting that the compound was transformed into some other substance(s), presumably with its nucleotide moiety going into nucleic acids and the peptide moiety going into some essential proteins.

3. Another noteworthy phenomenon observed during the process of cell division was the incorporation of ^{35}S in a group of hot PCA-soluble substances. These sulfur substances were revealed to be sulfur-containing nucleotidic compounds, which might possibly be some essential components of, or substances in close relation to, deoxypentose nucleic acid (DNA).

Plant and Cell Physiol., **1**, 131–142 (1960)

Role of Sulfur in the Cell Division of *Chlorella*, with Special Reference to the Sulfur Compounds Appearing during the Process of Cell Division. I

By Eiji HASE, Sayoko MIHARA and Hiroshi TAMIYA

Division of Biosynthesis I

1. The role of sulfur in the cell division of *Chlorella* was studied by following the fate of the sulfur supplied to the sulfur-deficient cells using ^{35}S as a tracer.

3. The sulfur-deficient cells which were unable to perform cell division were made capable of division by the provisions of ^{35}S-labeled sulfate under non-photosynthesizing conditions. Soon after the provision of sulfate the labeled sulfur went rapidly into the cold perchloric acid (PCA)-soluble fraction of algal cells, almost entirely in the form of sulfate and/or some other inorganic sulfur substance(s). With the lapse of time, more or less remarkable changes occurred in the pattern of ^{35}S-distribution in different fractions of cell material. It was noticed that, at the

Plant and Cell Physiol., **2**, 9–24 (1961)

Role of Sulfur in the Cell Division of *Chlorella*, with Special Reference to the Sulfur Compounds Appearing during the Process of Cell Division. II

By Eiji HASE, Sayoko MIHARA and Hiroshi TAMIYA

Division of Biosynthesis I

1. *Chlorella* cells, which had been grown synchronously under sulfur-deficient conditions and thus rendered unable to perform cell division, were made capable of nuclear and cellular division by being supplied with ^{35}S-labeled sulfate and nitrate under photosynthesizing conditions, and the fate of sulfur during these recovery processes was followed.

2. When the S-starved cells were provided with sulfate alone under photosynthesizing conditions, cells grew appreciably in mass performing nuclear division but remained incapable of cellular division. During these processes most of the ^{35}S was found to be incorporated into the protein fraction of algal cells.

3. When the cells which had been stalemated

at the above-mentioned stage were supplied with nitrate, they grew further in mass and eventually performed cellular division. During this period the ^{35}S was found to be distributed not only in the protein fraction, but also in an appreciable amount in the cold and hot acid-soluble fractions.

4. By paper-electrophoretic experiments it was found that the nature of the sulfur substances appearing in the hot acid-soluble fraction changed strikingly during the process of cellular division. Zone electrophoresis and an anion-exchange chromatography of these substances isolated from the cells at the completion of cellular division, disclosed that they were most probably deoxypentose polynucleotides containing sulfur in some form yet unidentified.

5. It was demonstrated that there exist some antagonistic relations between the protein synthesis and the formation of these sulfur-containing deoxypentose polynucleotides, and that the former predominates under photosynthesizing conditions, the latter under non-photosynthesizing conditions.

Radioisotopes in Scientific Research, **4**, 432-438 (1958)

Effect of Some Poisons upon the Mechanism of Photosynthesis as Studied by Technique of 'Pre-Illuminations' Using C^{14} as a Tracer

By Hiroshi Tamiya, Shigetoh Miyachi, T. Hirokawa and T. Katoh

Division of Biosynthesis I

It has been shown by Calvin *et al.* (1, 2) that the CO_2 fixing capacity of green cells increased markedly when the cells were illuminated prior to the provision of CO_2 in the dark. The agent responsible for the enhanced CO_2-fixation was concluded by Tamiya *et al.* (8) to be a certain reducing substance(s) (designated by R) which is formed photochemically from its precursor (P) with liberation of oxygen:

$$P + H_2O \xrightarrow{\text{light}} R + 1/2\,O_2 \qquad (I)$$

In the absence of CO_2, R is re-oxidized by P by reacting with oxygen:

$$R + O_2 \longrightarrow P + H_2O_2 \qquad (II)$$

the resulting H_2O_2 being immediately decomposed by the catalase existing in chloroplasts:

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + 1/2\,O_2 \qquad (III)$$

In normal photosynthesis, R reacts, not with O_2, but with phosphoglyceric acid (PGA, which is formed from CO_2 and ribulose-diphosphate, RDP) to give rise to triosephosphate, which in turn is eventually converted into RDP (13). Thus the overall reaction between R and CO_2 proceeds in the manner:

$$R + 3/5\,CO_2 \longrightarrow P + 1/5\,PGA \qquad (IV)$$

Using *Chlorella* as material, the effects of cyanide, azide and hydroxylamine upon the above-mentioned four reactions were investigated. It was found that cyanide inhibits reactions (III) and (IV) leaving (I) and (II) intact, while azide and hydroxylamine affect all four reactions with relative strengths which vary more or less markedly according to the temperature.

It was thus revealed by the tracer technique that the mechanism of inhibition of photosynthesis caused by these substances is far more complicated than it has been generally assumed heretofore.

Biochim. Biophys. Acta, **46**, 200-202 (1961)

Some Observations on the Phosphorus Metabolism in Growing *Chlorella* Cells

By Shigetoh Miyachi and Hiroshi Tamiya

Division of Biosynthesis I

Preliminary Note.

Chem. and Pharm. Bull., **8**, 46 (1960)

Steroid Studies. XV. Synthesis of 4, 4-Dimethylcholecalciferol and Its Conversion into 10-Isosteroid

By Eiji Ohki

Division of Chemistry

In order to examine the vitamin D activity, 4, 4-dimethyl-cholecalciferol (I), m.p. 71-73°, $[\alpha]_D$ +23.7°, was prepared by irradiation of ultraviolet rays on 4, 4-dimethyl-7-dehydrocholesterol. Pyrolysis of reaction of (I) was found to result in preferential cyclization to pyrocalciferol-type compound and 4, 4-dimethyl-10-isocholesta-5, 7-dien-3β-ol, m.p. 71-73°, $[\alpha]_D$+543°, was obtained in a good yield.

J. Am. Chem. Soc., **82**, 1442 (1960)

Steroid Studies. XVI. Isolation of 22-Dehydrocholesterol from *Hypnea japonica*

By Kyosuke Tsuda, Kiyoshi Sakai, Katsumi Tanabe and Yukichi Kishida

Division of Chemistry

22-Dehydrocholesterol was isolated from a species of red algae, *Hypnea japonica*.

J. Am. Chem. Soc., **82**, 3396 (1960)

Steroid Studies. XVII. On the Absolute Configuration of C-24-Ethyl of Stigmasterol

By Kyosuke Tsuda, Yukichi Kishida and Ryoichi Hayatsu

Division of Chemistry

The ozonolysis product of *i*-stigmasterylmethylether, 2-ethyl-3-methylbutanal (+) was converted throught the corresponding alcohol (−) and its tosylate (−) to the reference compound, methylethylisopropylmethane (−) and the absolute configuration of the C-24-ethyl group of the stigmasterol series was thus shown to have the C-24α configuration in Plattner's convention. Further, some evidence on the configuration of the C-24-methyl of the ergosterol series is presented.

Chem. and Industry, 1411 (1959)

Absolute Configuration of C-(24)Ethyl of Stigmasterol

By Kyosuke Tsuda, Ryoichi Hayatsu and Yukichi Kichida

Division of Chemistry

The ozonolysis product of *i*-stigmasterylmethylether, 2-ethyl-3-methylbutanal (+) was converted through the corresponding alcohol (−) and its tosylate (−) to the reference compound, methylethylisopropylmethane (−) and the absolute configuration of the C-24-ethyl group of the stigmasterol series was thus shown to have the C-24α configuration in Plattner's convention. Further, some evidence on the configuration of the C-24-methyl of the ergosterol series is presented.

Chem. and Pharm. Bull., **8**, 229 (1960)

Steroid Studies. XVIII. Reduction of 5, 7-Diene in 10-Isosteroid

By Eiji Ohki

Division of Chemistry

The behavior of the double bond in the various derivatives of 4, 4-dimethyl-10-isocholesta-5, 7-dien-3β-ol to reduction with sodium and ethanol or over Raney nickel catalyst was comparatively examined with that of 10-normal steroid. It was thereby found that the double bond at 7-position in the iso series is easily reduced by catalytic reduction over Raney nickel.

Chem. and Pharm. Bull., **8**, 554 (1960)

Untersuchungen über Steroide. XX. Die Sterine aus grünen Meeres-Algen

Von Kyosuke Tsuda und Kiyoshi Sakai

Die Abteilung der Chemie

Im Zusammenhang mit den früheren Untersuchunger über Algen-Sterine wurden diesmal die Sterin-Bestandteile der grünen Meeres-Algen untersucht. Aus *Monostroma nitidum* Wittrock wurden nämlich das Triterpenoid, Friedelin und ein Sterin, das vermutlich das Haliclonasterin was, isoliert. Bei der Aufarbeitung des Sterin-Bestandteils von *Enteromorfa linza* L. ergab sich ein Stigmastadienol, dessen Eigenschaften mit denjenigen des Δ^5-Avenasterins identisch waren. Der Sterin-Gehalt der grünen Alge war kleiner als derjenige der roten bzw. branuen Alge.

Chem. and Pharm. Bull., **8**, 1128 (1960)

Steroid Studies. XXI. On the Reaction of 5α, 8α-Ethylenic Steroids

By Kyosuke Tsuda and Shigeo Nozoe

Division of Chemistry

Diels-Alder reaction of methyl acrylate and acrolein on cholesta-5, 7-dien-3β-ol acetate was carried out and the structure of the adduct thereby obtained was determined. Solvolysis of 3-methanesulfonate of these 5α, 8α-ethylene steroids was carried out and retention of configuration was found to take place, whose mechanism was considered.

Chem. and Pharm. Bull., **9**, 131 (1961)

Steroid Studies. XXVII. A New Aromatization Reaction of the Homoannular Diene System. (I). On the Structure of the Phenolic Compound derived from 3β-Acetoxylanosta-5, 8-dien-7-one

By Kyosuke Tsuda, Eiji Ohki, Junnosuke Suzuki and Hiroshi Shimizu

Division of Chemistry

It was observed that the methyl group in the 19-position of 3β-acetoxylanosta-5, 8-dien-7-one (I) would be easily eliminated as methane and the B-ring was aromatized by treatment with zinc. The other chemical and photochemical behaviors of (I) were examined.

Chem. and Pharm. Bull., **8**, 626 (1960)

Mikrobiologische Hydroxylierung der Steroide. X. Über 7β, 15β-Dihydroxypregn-4-en-3, 20-dion

Von Kyosuke Tsuda, Toshinobu Asai, Yoshihiro Sato, Tokuji Tanaka, Takemasa Matsuhisa und Hiroko Hasegawa

Die Abteilung der Chemie

Das Dihydroxyprogestern, welches von diesen Autoren durch Einwirkung von *Syncephalastrum racemosum* auf Progesteron (I) einst gewonnen wurde und damals nach dem Strukturzeichen von 7α, 15β-Dihydroxyprogesteron genannt wurde, ergibt sich diesmal bei der Umsetzung von (I) sowie von 7β-Hydroxyprogesteron mit *Diplodia tubericola*. Aus diesen Tatsachen sowie aus den Ergebnissen der vorherigen Mitteilungen ist ersichtlich, dass das richtige Strukturzeichen des Dihydroxyprogesterons 7β, 15β-Dihydroxyprogesteron ist.

Chem. and Pharm. Bull., **8**, 1056 (1960)

Mikrobiologische Verwandlung von Thebain in das 14-Hydroxycodeinon mittels einiger Pilze

Von Kazuaki IIZUKA, Shigenobu OKUDA, Kō AIDA, Toshinobu ASAI, Kyosuke TSUDA, Michihiro YAMADA und Isawo SEKI

Die Abteilung der Chemie

Thebain (I) wurde durch Fermentierung mit *Trametes sanguinea* in 14β-Hydroxycodeinon (45.7%) und 14β-Hydroxy-codein übergeführt. Das letztere konnte auch durch mikrobiologische Umwandlung von (I) mit *Pleurotus ostreatus* erhalten werden.

Chem. and Pharm. Bull., **8**, 257 (1960)

Untersuchungen der Eierstockextrakte von Kugelfischen. XI. Über Tetrodotoxin. (2)

Von Kyosuke TSUDA, Masaaki KAWAMURA und Ryoichi HAYATSU

Die Abteilung der Chemie

Tetrodotoxin, $C_{12}H_{19}O_9N_3$, gab mit Essigsäureanhydrid und Pyridin das Acetat-A-Chlorohydrat, $C_{11}H_{14}O_7N_2(COCH_3)_2 \cdot HCl$. Durch Natriumborhydrid-Reduktion von Acetat-Chlorohydrat und darauf-folgender Tosylierung entstand ein Produkt der Summenformel $C_5H_7ON(C_7H_7O_2S)_2$, dessen Dihydro-Derivat nach Lithiumaluminium-hydrid-Reduktion das bekannte β-Methylpyrrolidin, lieferte.

J. Org. Chem., **25**, 783 (1960)

The Chemistry of the Spiroaminoketal Side Chain of Solasodine and Tomatidine. I. Improved Preparation of 3β-Acetoxy-5, 16-Pregnadien-20-one and 3β-Acetoxy-5α-pregn-16-en-20-one from Solasodine and Tomatidine

By Yoshio SATO, Nobuo IKEKAWA and Erich MOSETTIG

Division of Chemistry

The steroidal alkaloids solasodine and tomatidine have been degraded in excellent over-all yields (65–68%) to 3β-acetoxy-5, 16-pregnadien-20-one and 3β-acetoxy-5α-pregn-16-en-20-one by conversion of the O, N-diacetates of the alkaloids into the respective pseudoacetylamino derivatives, chromic anhydride oxidation of the latter and final hydrolysis with acetic acid.

J. Org. Chem., **25**, 786 (1960)

The Chemistry of the Spiroaminoketal Side Chain of Solasodine and Tomatidine. II. Chemistry of 3β, 16β-Diacetoxy-20-(2′-$\Delta^{2'}$-N-acetyl-5′-methyltetrahydropyridyl)-5-pregnene

By Yoshio SATO and Nobuo IKEKAWA

Division of Chemistry

The chemistry and the manifold interrelationship of the acetylated tetrahydropyridyl pregnenes and the diacetylamino-furostadiene derivative obtained in the treatment of solasodine with acetic anhydride are discussed.

J. Org. Chem., **25**, 789 (1960)

The Chemistry of the Spiroaminoketal Side Chain of Salasodine and Tomatidine. III. The Reaction of O, N-Diacetylsolasodine in Acidic Media

By Yoshio Sato and Nobuo Ikekawa

Division of Chemistry

The isomerization of O, N-diacetylsolasodine in nonpolar media forms the pseudo derivative, 26-acetylamino-5, 20(22)-furostadien-3β-ol acetate in good yields. Aqueous or alcoholic media promote the formation of C-22 substituted 26-acetylaminofurostene derivatives. The chemistry of the 22-hemiketal and alkoxyketals is discussed.

J. Org. Chem., **25**, 1962 (1960)

The Chemistry of the Spiroaminoketal Side Chain of Solasodine and Tomatidine. IV. Chemistry of the Tomatidine Side Chain

By Yoshio Sato, H. George Latham, Jr. and Nobuo Ikekawa

Division of Chemistry

Treatment of tomatidine with acetic anhydride yields an acetylated Δ^2-tetrahydropyridylallopregnane and a diacetylamino-5α, 20(22)-furostene derivative. With a zinc chloride-acetic anhydride-acetic acid solution tomatidine affords a Δ^1-tetrahydropyridylallopregnane derivative. The chemistry of these compounds is discussed.

J. Org. Chem., **26**, 628 (1961)

Gas Chromatographic Separation of Steroidal Amines

By W. J. A. Vandenheuvel, E. C. Horning, Yoshio Sato and Nobuo Ikekawa

Division of Chemistry

This study was made of the gas chromatographic behavior of a series of steroidal amines derived from tomatidine and solasodine, and the relationships between stereochemical assignments and retention times of these compounds were correlated.

J. Agr. Chem. Soc. Japan, **33**, No. 7 (1959)

Studies on the Volume Fraction of Cells in Snspension

By Shozo Koga, Chie Kuribayashi and Kazuko Nunomura

Division of Biophysics

A rheological method for the evaluation of volume fraction (ϕ) of cells in suspension was discussed with special regard to *Saccharomyces cervisiae*. Experimental results are well fitted by a quadratic equation of ϕ, as expected in accordance with Einstein's formula for simple, rigid and spherical particles in suspension. The coefficients in the equation seemed, however, to take values somewhat different from the ones theoretically computed. As examples of direct application of the rheological method, growth curves and osmotic volume changes were given, showing, thereby, 1.2×10^{-1} mol/l as the equivalent osmotic pressure of the yeast cell.

J. Agr. Chem. Soc. Japan, **33**, No. 11 (1959)

Studies on Light Scattering by Cell Suspensions

By Shozo Koga and Teruyuki Fujita

Division of Biophysics

By use of the three methods proposed for the evaluation of the cellular refractive index, unicellular organisms in aqueous suspensions were found to be "soft scatters" with relative index of refraction 1.04~1.05. As shown for *Saccharomyces cerevisiae*, *Rhodotorula glutinis* and *Micrococcus sphaeroides*, the total scattering cross section of a single spherical cell was given by Rayleigh-Gan's or Hart-Montroll's formulae as function of its diameter, index of refraction and the wave-length of incident light. Some remarks were, thereby, made in relation to the microbiological application of turbidimetry.

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